THE BINDING OF DIVALENT CATIONS TO TOBACCO MOSAIC VIRUS AND TO SOME ISOMETRIC PLANT VIRUSES

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SUMMARY

The binding of divalent cations (particularly calcium and magnesium) to strains of tobacco mosaic virus (TMV) and their isolated proteins was investigated, using equilibrium dialysis and potentiometric titration, in an attempt to elucidate the role of divalent cations in virus stabilisation. It was found that dissociation of bound calcium ions from TMV is apparently a necessary but insufficient condition for *in vitro* virus disassembly. TMV and the closely related strain, Y-TAMV, possessed three groups per protein subunit which titrated near neutral pH and which showed significant metal ion binding. The tightest of the three calcium binding sites, which was absent on the RNA-free protein, had a computed pK_H of 8.3 and pK_{Ca} of 5.2 and had a significantly higher affinity for Ca^{2+} over Mg^{2+}. This group thus had some of the characteristics to be expected for a calcium-mediated switch controlling *in vivo* virus disassembly, and possibly controlled the *in vitro* alkaline degradation of TMV as well. Both the U2 and cowpea strains of TMV bound one additional metal ion per protein subunit relative to vulgare, this binding site being retained by the polymerised proteins. However, calcium ions stabilised the polymerised forms of the proteins of all four TMV strains at pH values where depolymerisation would normally have occurred.

Both bromegrass mosaic virus and turnip crinkle virus bound calcium ions, which stabilised compact forms of these viruses. The phenomenon of cation binding is thus not limited to TMV. In the light of published evidence, it appears that most if not all plant viruses are able to bind divalent cations, which thus represent a hitherto disregarded stabilising element.

CHAPTER ONE

INTRODUCTION

The presence of small but significant amounts of divalent cations, particularly calcium and magnesium, in preparations of purified tobacco mosaic virus (TMV) has been recognised since the early work of Loring and his colleagues in the late 1950's. Although subsequent analyses by the same and other workers differed regarding the absolute amounts of these cations bound per TMV particle, which depended on the method of virus purification employed, a portion thereof appeared to resist all attempts at removal including chelation by sequestering agents with a high affinity for these cations. These latter observations were subsequently questioned by some workers, but it was clear that at least one divalent cation per every two protein subunits was bound to TMV. This binding, apparently to a site involving the RNA, had a sufficiently high affinity for the cation to be regarded as an intrinsic component of the purified virus.

The precise function of these bound cations has remained obscure, and as recently as 1975 in his book "The Chemistry of Viruses" Knight stated that "...it is doubtful if the remaining few atoms of tightly bound metal are of crucial importance". That their presence was not entirely without effect was recognised in the early 1970's in Lauffer's laboratory in Pennsylvania. It was found that TMV exhibited a negligible Donnan effect, which could be increased to the value expected on theoretical grounds by the removal of the bound cations using dialysis against a chelating agent. The presence of bound divalent cations thus markedly affected the charge on the TMV virions. A further effect of particularly bound calcium ions was later reported from the same laboratory. Isolated tobacco mosaic virus protein (TMVP), which has been polymerised into helical rods by acidification to pH 5.5, can be induced to depolymerise within minutes at pH 6.5 by cooling from 25°C to 5°C.

However, if the initial polymerisation occurs in the presence of Ca^{2+} ions, the polymers are measurably stabilised against the temperature-induced depolymerisation. This effect was claimed to be due to the binding of calcium to, and the resultant stabilisation of, a form of the polymer that existed only below pH 6.

A further stabilising effect of divalent cations was reported in the early 1970's from Brakke's laboratory in Nebraska. Following the observation by Lauffer and his colleagues that alkaline degradation of TMV was favoured by EDTA, Brakke and coworkers observed that the degradation of TMV at pH 9 was inhibited by divalent cations. These cations apparently affected the *in vitro* separation of the protein from the nucleic acid, resulting in the formation of partially-stripped rods of varying lengths. Tobacco mosaic virus dissociated completely to free RNA and protein in the presence of bentonite at pH 9, but the presence of cations stopped the stripping process at certain sites along the RNA. Divalent cations were at least a thousand-fold more effective in their protective action than were monovalent cations, and it was postulated that TMV might require a proper pH, concentration of divalent cation, and a bentonite-like material to identify the correct part of the cell for disassembly into protein and RNA.

The development of this idea was taken a stage further in the early 1970's by Durham and his colleagues working in Klug's laboratory in Cambridge, U.K.. After potentiometric titration of both intact virus and isolated protein, they concluded that TMV contains two abnormally titrating groups per protein subunit. Earlier, Caspar had proposed the presence of carboxyl groups with raised pK_H values to explain his observations regarding the binding of lead to TMV. Durham and coworkers reasoned that the lead-binding, raised pK_H carboxyls in TMV also served to bind calcium ions, by analogy with certain calcium ionophores and calcium-binding proteins which possess carboxyl groups having raised pK_H values. Dissociation of these bound calcium ions from

TMV _in vivo_ due to the low calcium concentration of the cytoplasm could then destabilise the virus and serve as a trigger for the intracellular uncoating of TMV.

The present study attempts to verify the hypothesis that dissociation of bound calcium ions from the TMV particle soon after infection initiates the separation of the protein subunits from the nucleic acid strand. During the course of the investigation it became evident that dissociation of calcium ions from the virion near neutral pH could not by itself result in disassembly of the virus particle, and that various other factors, probably acting in concert with the low calcium concentration of the cytoplasm, had to be involved. However, since divalent cations are known to influence such phenomena as the alkaline degradation of TMV, the depolymerisation of TMVP, and the capsid stabilisation of certain spherical plant viruses, the role of divalent cation binding in maintaining the structure of various viruses and strains was investigated.

CHAPTER TWO

LITERATURE REVIEW

A. Viruses

1. Tobacco mosaic virus (type strain)

Tobacco mosaic virus (TMV) is a ubiquitous plant virus, infecting a wide range of herbaceous hosts (Zaitlin & Israel, 1975). It was first isolated in a paracrystalline form by Stanley (1935), and has subsequently been the object of a wide range of biological, biochemical and biophysical investigations. Today it ranks as one of the best-characterised biological macromolecules, about which a number of review articles have been written (Fraenkel-Conrat & Ramachandran, 1959; Anderer, 1963; Caspar, 1963; Lauffer & Stevens, 1968; Butler & Durham, 1977).

(a) TMV structure

The structure of the TMV particle has been reviewed by Anderer (1963), Caspar (1963), and Lauffer & Stevens (1968). The virion is rod-shaped and consists of a single RNA strand intercalated between the turns of a helical assemblage of protein subunits. From the amino acid composition, each protein subunit is known to have a molecular weight of 17 500 daltons, while biophysical measurements indicate that the virion has a particle weight of 40×10^6 daltons. The RNA content of TMV has been established by chemical methods to be 5.1%, from which one can calculate that the RNA has a molecular weight of about 2×10^6 daltons. This figure has been confirmed by direct biophysical measurements on the extracted virus RNA. From the above data it follows that each TMV particle thus contains about 2 130 protein subunits, each subunit being in contact with 3 nucleotides of the RNA.

It has been shown by electron microscopy and X-ray crystallography that the virus particle is a rigid, hollow cylinder with a length of about 3000 Å and a maximum diameter of 180 Å, and that the RNA is situated in the particle at a radius of 40 Å. The axial hole has a diameter of 40 Å and is penetrated by electron-dense stains, indicating that the interior of the particle is in contact with the aqueous environment.

(b) Structure of the protein subunit

Each tobacco mosaic virus protein (TMVP) subunit occupies a sector-shaped domain in the virus structure and has maximum dimensions of about 70 x 25 x 23 Å (Klug & Caspar, 1960). Although TMVP normally polymerises in neutral aqueous media (Durham, 1972a), sedimentation constants of the monomer have been determined in denaturing solvents. Schachman & Hersh (quoted by Fraenkel-Conrat & Singer, 1954) found that the TMVP monomer sedimented at 1.2 S in 1% sodium dodecyl sulphate, while Buzzell (1960) obtained a value of 2.0 S for TMVP in 6 M urea. Budzynski & Means (1971) found that TMVP sedimented at 1.4 S after they had prevented polymerisation by modifying 8 carboxyl groups per subunit with ethylenediamine. Ansevin & Lauffer (1959) produced a component with a sedimentation constant of 1.9 S by lowering the concentration to 0.01% at 3°C, while a sedimentation constant of 2 S was observed by Wittmann (1959) with 0.5% TMVP in 0.1 N NaOH at 20°C.

The subunit contains 158 amino acid residues of which the precise sequence in the polypeptide chain is known (Anderer _et al._, 1960; Tsugita _et al._, 1960). Using information obtained primarily from amino acid sequencing, X-ray crystallography, electron microscopy, and studies on reactivity of certain residues, Durham & Butler (1975) predicted a structure for TMVP. Their positioning of the various regions of the polypeptide chain bore many features in common with that subsequently determined by X-ray crystallography of the virus (Holmes _et al._, 1975) and of the protein disc (Champness _et al._, 1976). The most important features to emerge from those studies were that the carboxyl terminal end, and probably also the amino terminal end, of the polypeptide chain was situated at the surface of the particle and was therefore exposed to the environment; that the polypeptide chain had a "paper-clip" structure consisting of four extended, α-helical, radially-orientated regions (roughly, residues 20-30, 40-60, 80-90 and 120-140) connecting the domains at high radius with those at low radius; that three regions of the chain (circa residues 40, 90 and 120) were situated close to the RNA at 40 Å radius; and that the region in the proximity of residue 110 was closest to the central axial hole at 20 Å radius.

Attempts to determine the precise location of certain amino acids, particularly their surface availability, have been reviewed by Durham & Butler (1975). These attempts have involved using X-ray crystallography to determine the radial positions of amino acids to which heavy atoms have been attached, or using enzymes, antibodies or chemical reagents to infer from their reactivity which residues are exposed, either in the virion or in the dissociated protein. Of particular note are the arginine residues at positions 90, 92 and 113, which are conserved in a number of TMV strains (Wittmann-Liebold & Wittmann, 1967), and which probably serve to neutralise the negative charges on the RNA phosphate groups (Durham & Butler, 1975). It is also evident that the charged residues are concentrated between 20-40 Å radius and between 70-90 Å radius (i.e. at or near the extremities of the molecule which are normally in contact with solvent), while uncharged and hydrophobic residues occur in the central domain, between 40 and 70 Å radius, which is the region involved in inter-subunit bonding.

(c) Cations bound to purified TMV

The presence of bound cations in purified preparations of TMV was first recognised by Loring & Waritz (1957), who reported that TMV in 0.1 M phosphate buffer pH 7 contained about 300 calcium ions, about 1 000 magnesium ions, about 15 copper ions and about 20 iron ions bound per virion of

40×10^6 daltons. These ions appeared to be located in the nucleic acid rather than in the protein, and, while iron and copper were largely unaffected, the calcium and magnesium contents were reduced by more than 90% by prolonged dialysis against EDTA. Calcium and magnesium were found by Loring et al. (1962) to be the cations complexed to TMV in the largest amounts. TMV, that had been purified without the addition of buffer and had been resuspended in deionised water after each of three or four centrifugation cycles, contained about 3 400 atoms of calcium and about 1 000 atoms of magnesium per virion of 40×10^6 daltons, in addition to smaller quantities of sodium, potassium, iron and manganese. The total complement of bound cations was equivalent to about 8 800 negatively-charged binding sites per particle of 40×10^6 daltons, which Loring et al. (1962) calculated to be of the same order of magnitude as the number of acidic groups on the protein not neutralised by basic groups on the protein or RNA, i.e. the majority of free acidic groups present on the protein bound calcium or magnesium. McMichael (1973), however, pointed out that the analysis of Loring et al. (1962) included counter ions as well as bound ions, and he applied data obtained from the titration curves of Scheele & Lauffer (1967) to calculate the probable number of counterions per particle. He concluded that of the approximately 8 900 positive charges that could be ascribed to cations, about 2 500 were due to cations actually bound by the TMV particle. This would have corresponded to roughly one divalent cation bound per two protein subunits.

The analyses of Loring & Waritz (1957) were confirmed by Wacker et al. (1963), who found that certain metals, particularly calcium and magnesium, were intrinsic components of TMV and TMV-RNA. After dialysis against EDTA, the content of residual firmly-bound cations in the virus was comparable to that contained in extracted TMV-RNA. These authors interpreted their results as meaning that metal was bound to TMV in two different ways: by relatively loose binding to the protein moiety, and also by binding to the RNA so strongly as to resist removal by EDTA. The firmness of binding precluded the involvement of RNA phosphate groups, and suggested chelation by the nitrogenous bases.

The binding of additional K^+ and Ca^{2+} to TMV and TMVP was investigated by Shalaby et al. (1968), who found that at pH 4.3 (the isoionic point) neither added potassium nor added calcium were bound by intact TMV or TMVP. At pH 9.0, 2.5 K^+ and 1.8 Ca^{2+} ions were bound per protein subunit by TMV, while no K^+ or Ca^{2+} was bound by TMVP, indicating that the RNA was possibly involved in the binding by the virus. McMichael (1973) questioned the results of Shalaby et al. (1968), as he found that an average of 2 Ca^{2+} ions per subunit could be bound by TMVP at pH 6.5. He proposed the existence of two forms of TMVP which could exist at pH 6.5: a tightly packed structure (possibly helical) induced by acidification to below pH 6, capable of binding cations which then stabilised the structure at pH 6.5, and a second, more loosely-packed structure not capable of binding cations, produced either by raising the pH of a TMVP solution not containing cations from pH 5.5 to pH 6.5 or by polymerisation of TMVP at pH 6.5 in the absence or presence of cations (McMichael & Lauffer, 1975).

Indirect evidence for the binding of cations to TMV and TMV-RNA was obtained by Adiarte & Lauffer (1973), who found that TMV-RNA in phosphate buffer at pH 7.5, and TMV in phosphate buffer at pH values ranging from 5.5 to 8.0, exhibited less than 1% of the expected Donnan effect. Reconstituted TMV, or TMV dialysed against EDTA, did exhibit the expected Donnan effect, which, in the case of reconstituted TMV, could be abolished by the addition of calcium ions.

(d) Degradation of TMV by alkali

TMV is recognised as being a particularly stable plant virus (Zaitlin & Israel, 1975). One of the earliest recognised methods of degrading TMV was by means of alkali (Schramm et al., 1955; Harrington & Schachman, 1956), which resulted in the production of soluble A-protein ("alkalischer protein"). It was found that many TMV particles, however, seemed partially resistant to alkali, only degrading two-thirds of their length and leaving an apparently alkali-resistant 100 nm rod (Schramm _et al._, 1955; Harrington & Schachman, 1956; Perham, 1969). A stable fraction, constituting approximately 25% of the population, appeared to resist degradation (Schramm _et al._, 1955; Perham, 1969). Five stable intermediates were formed during the stripping process (Perham, 1969), which Onda _et al._ (1970) have claimed proceeds from the 3'-end of the RNA, and Perham & Wilson (1976) have claimed proceeds from the 5'-end. Alkaline degradation of TMV was favoured by EDTA (Shalaby & Lauffer, 1967) and hindered by certain basic proteins (Diener & Desjardins, 1966; Taniguchi _et al._, 1967).

The effect of bentonite on the alkaline degradation of TMV was investigated by Brakke & van Pelt (1969), who found that degradation in tris buffer at pH 9 was increased by bentonite, and reduced but not prevented by 10^{-2} to 10^{-4} M concentrations of magnesium or polyamines. If EDTA buffers were used, degradation of TMV occurred at pH 7.0 and above in the presence of bentonite, and at pH 8.0 and above in the absence of bentonite. The action of bentonite in the alkaline degradation of TMV was further investigated by Brakke (1971), who concluded that TMV protein was not adsorbed by the bentonite, although BMV protein apparently was. The presence of 0.1 M NaCl in addition to the bentonite resulted in incomplete degradation to short, variable length rods only and not to RNA and protein.

The protection of TMV by cations against alkaline degradation, reported by Brakke & van Pelt (1969) and Brakke (1971), was examined by Powell (1975), who reported that their effect was to stop the stripping process at certain sites along the nucleic acid. The protection of TMV occurred at 10^{-3} to 1.0 M concentrations of monovalent cations, and at 10^{-6} to 10^{-3} M concentrations of divalent cations. Powell favoured a mechanism whereby the cation linked the protein to the nucleic acid, and he excluded the phosphate groups of the nucleic acid as the binding sites. He postulated that TMV might require the correct pH, a certain concentration of free magnesium or other ions, and a bentonite-like material in order to start dissociating in a particular part of the cell.

(e) Assembly properties of TMVP

(i) Early observations

Knight & Lauffer (1942) were first to report that nucleic acid-free protein, obtained by alkaline degradation of TMV, tended to aggregate to form rapidly sedimenting particles. Schramm (1943) showed that such A-protein could aggregate to form virus-like rods resembling TMV. The structure of these rods was shown by Franklin (1955) to be helical, similar to that of intact virus except for the absence of RNA. However, Franklin & Commoner (1955) obtained X-ray diffraction patterns for polymerised protein, which indicated that, under some circumstances, an alternative, non-helical, "stacked-disk" structure was formed by TMVP. It was recognised that, in addition to the A-protein sedimenting at about 4 S (Harrington & Schachman, 1956) and the protein rods sedimenting at >100 S, a number of stable intermediates existed with sedimentation constants of about 8.5 S, 20 S, 30 S and 50 S (Schramm, 1947; Schramm & Zillig, 1955). These 20-50 S components observed in the electron microscope consisted of hollow disks having the same diameter as the TMV particle, but which varied in thickness from 50-100 Å. Below pH 6.3 these disks aggregated to form long protein rods.

(ii) Structures of the various TMVP aggregates

Since the early structural investigations of the long TMVP rods by Franklin (1955) and Franklin & Commoner (1955), numerous other attempts have been made to elucidate the structures of the various TMVP aggregates. The 4 S A-protein was shown by Banerjee & Lauffer (1966) to be a trimer. The disks of TMVP are built up of rings of TMVP subunits, each ring containing 17 subunits (Finch *et al.*, 1966; Crowther & Amos, 1971) although some authors have claimed that there are 16 subunits per ring (Nixon & Woods, 1960; Markham et al., 1963). The 20 S and 30 S aggregates were shown by Caspar (1963), on theoretical grounds, to contain two and four such rings respectively, which prediction was subsequently confirmed by Durham & Finch (1972).

The long stacked-disk rod, which normally had a sedimentation constant in excess of 150 S and was known to differ structurally from the long helical rods (Franklin & Commoner, 1955), was first recognised by Klug & Caspar (1960) on the electron micrographs of Nixon & Woods (1960), and again by Markham et al. (1964). The conditions required for their formation, however, were first reported by Carpenter (1970), who found that TMVP polymerised slowly to stacked-disk rods in alkaline solutions of pH 8-10.5. He could not produce stacked disks by acidifying TMVP, nor could stacked disks be depolymerised by acidification unless the pH was reduced to 2. The depolymerised protein thus obtained was subsequently unable to reaggregate to form helices, indicating that protein obtained from stacked disks differed from that obtained from protein helices, as the latter could be repeatedly polymerised and depolymerised simply by pH adjustment. The requirement of alkalinity for the formation of stacked-disk rods was confirmed by Durham & Finch (1972), who stated that the existence of stacked-disk rods in acid solutions arose from pre-existing stacks of disks which were present when the solution was alkaline. It was subsequently reported by Durham (1972b) that stacked-disk rods were comprised of TMVP subunits that had undergone a cleavage in the polypeptide chain.

(iii) Factors controlling TMVP polymerisation

The aggregation of TMVP was found to be accelerated by a rise in temperature (Knight & Lauffer, 1942; Harrington & Schachman, 1956; Lauffer et al., 1958). Lauffer et al. (1958) also showed that, whereas at pH 6.5 the aggregation state depended on the temperature, at pH 5.0 TMVP existed only in the polymerised state and at pH 7.7 only in the depolymerised state. These authors concluded that the temperature dependence indicated a positive enthalpy for polymerisation, which, being spontaneous at 30°C, also involved a positive entropy change. They therefore postulated that the hydrated monomer lost water on polymerisation, the breaking of water-monomer bonds resulting in a gain in entropy.

Between 1963 and 1969 a series of twelve papers emerged from Lauffer's laboratory in Pittsburgh, mainly attempting to elucidate the role of water and of hydrophobic bonding on the polymerisation of TMVP. Although they investigated in detail many of the physico-chemical aspects of TMVP polymerisation, it was not until the appearance of a series of papers from Klug's laboratory in Cambridge (Durham, 1972a; Durham & Finch, 1972; Durham & Klug, 1972; Butler et al., 1972; Butler & Durham, 1972) that the conditions of pH and ionic strength favouring the formation of each type of aggregate were clarified. An important feature to emerge from this work was that at pH 7.0 and ionic strength I=0.1 the natural state of aggregation of TMVP was not the helix but the 20 S disk in equilibrium with 4 S A-protein. A rise in pH caused the disk to depolymerise to form A-protein, while lowering the pH caused the disks to aggregate to form the helix. Limited stacks of two or three disks could arise as intermediates during the disk-to-helix transition, or by raising the ionic strength of a solution of disks at pH 7.

The factor controlling the mode of aggregation of TMVP was shown by Butler et al. (1972) to be proton binding by two abnormally-titrating groups per TMVP subunit. TMV, which possesses no histidine or terminal amino group, has been shown by titration studies to contain two groups per subunit titrating with a pK_H close to 7 (Caspar, 1963; Ansevin et al., 1964; Scheele & Lauffer, 1967). It was suggested by Caspar (1963) that these were carboxylic acid groups which titrated abnormally because the helical structure constrained two pairs of carboxylate groups per subunit to be close to one another. The resultant electrostatic repulsion between them was reduced by the formation of two carboxyl-carboxylate pairs, each with a bound proton that now titrated near neutrality instead of pH 4. Studies on the binding of lead ions to TMV (Caspar, 1956; Fraenkel-Conrat & Narita, 1958) and the fact that two protons were released per subunit when the helix broke down (Caspar, 1963) supported the interpretation. Also, protons were bound when TMVP was induced to polymerise by raising the temperature (Ansevin et al., 1964).

An attempt was made by Butler & Durham (1972) to identify the abnormally-titrating groups by correlating the titration behaviour of five TMV variants with the conservation of critical amino acid residues (see section A2(b) below). These authors concluded that asp 115 and asp 116 comprised one probable pair, and that glu 145 was involved in the second. The positions of these residues in the TMVP structure (Champness et al., 1976) is in accordance with the radial positions of 25 Å and 84 Å from the particle axis determined by Caspar (1956) for the lead binding sites.

The carboxylic acid groups in the stacked-disk rods were found to titrate normally (Butler et al., 1972), which suggests that the carboxyl groups are not constrained to be as close as they are in the helix. The subunits in the disk have been shown to undergo a pairing interaction, in that the outer parts of the subunits in the two rings comprising a disk move towards each other in the axial direction (Durham et al., 1971), and Durham & Klug (1971) have suggested that this interaction is due to mutual repulsion by the charged groups. This repulsion could then be overcome either by lowering the pH to form a protonated carboxyl-carboxylate pair, or by the addition of RNA. In both cases the protein would then form a helix (see also section A1(f)).

(iv) Other factors influencing TMVP polymerisation

Various other factors, reviewed by Lauffer & Stevens (1968), have been found to influence the polymerisation of TMVP. A rise in temperature enhanced

TMVP aggregation at pH 6.5, but had little influence at pH 5.0 and pH 7.7 (Lauffer et al., 1958). When TMVP was caused to polymerise in an unbuffered medium by raising the temperature, protons were bound and the pH increased (Ansevin et al., 1964). Raising the ionic strength also induced polymerisation of TMVP (Khalil & Lauffer, 1967), as was later confirmed by Durham (1972a). Both the above-mentioned temperature and ionic strength effects are characteristic of entropic bonding between proteins, where hydrophobic regions in the subunits are juxtaposed (Lauffer, 1975). Polymerisation of TMVP was also enhanced by increasing the protein concentration (Banerjee & Lauffer, 1966). Depolymerisation induced by dilution of TMVP at pH 6.5 did not result in a drop in pH (Scheele & Lauffer, 1967), whereas depolymerisation induced by cooling did (Ansevin et al., 1964). Scheele & Lauffer (1967) concluded therefore that the temperature-induced polymerisation and the concentration-induced polymerisation were two different processes. This conclusion was subsequently challenged by Scheele & Schuster (1975) (see section A1(f)). Above pH 7.0 increasing the concentration of TMVP up to 20 mg/ml increased the concentration of 20 S discs only, the concentration of 4 S A-protein remaining constant at about 1 mg/ml (Taniguchi, 1969; Durham, 1972a). Various chemicals, such as KSCN, urea, thiourea, acetamide, EDTA, dioxane, sucrose (Lauffer & Stevens, 1968), sodium pyrophosphate (Lonchampt et al., 1972), and NaCl and KCl (Lebeurier et al., 1973) also influence TMVP polymerisation.

(v) Stabilisation of TMVP polymers by Ca^{2+} ions

Of particular relevance to this study was the observation that Ca^{2+} ions stabilised TMVP that had been acidified to below pH 6.0 in the presence of Ca^{2+}, against temperature-induced depolymerisation at pH 6.5 (McMichael, 1973; McMichael & Lauffer, 1975). On being cooled from 25°C to 5°C, TMVP in the absence of Ca^{2+} was totally depolymerised within 20 min, whereas in the presence of I=0.03 calcium, depolymerisation was incomplete after 12 h.

Magnesium or potassium did not retard depolymerisation. However, if the protein was then repolymerised in the presence of Ca^{2+} by raising the temperature at pH 6.5, the calcium did not retard subsequent depolymerisations. According to McMichael & Lauffer (1975) Ca^{2+} ions which are initially bound by the TMVP at pH 5.5 in the cold, stabilise polymerised TMVP at pH 6.5, and then dissociate irreversibly from TMVP during depolymerisation at pH 6.5. This explanation is, however, difficult to reconcile with an earlier finding in the same laboratory (Shalaby *et al.*, 1968) that calcium did not bind to TMVP between pH 4.5 and pH 9.5.

(f) Acid-base titration of TMV and TMVP

The presence of abnormally titrating groups in TMV was first recognised by Caspar & Caspar (quoted by Caspar, 1963). In TMVP these groups also titrated abnormally but co-operatively, since proton release occurred over a narrow range around pH 6.5 at 20°C. These groups were believed by Caspar (1963) both to be carboxyl-carboxylate pairs (see section A1(e)(iii) above).

The effect of charge on the polymerisation-depolymerisation of TMVP was investigated by Ansevin *et al.*(1964), who observed that there was a drop in pH when TMVP was depolymerised at pH 6.4 by lowering the temperature. A comparison of the titration curves of TMVP and intact virus showed a region of buffering near pH 6.0 to be present only in the protein curve, and confirmed that protons were dissociating from TMVP during polymerisation. They ascribed these protons to carboxyl groups, situated either on the mating faces of the subunits or within the helical grooves of the polymer, which titrated abnormally as a result of having been thrust by polymerisation into a medium of low dielectric constant. They also invoked this mechanism to explain the observation that, whereas the electrophoretic mobility of TMV remained constant above pH 5.0 (Kramer & Wittmann, 1958), the titration curve of TMV showed a steady increase in the amount of base neutralised up to pH 8.0. This study was extended in the same laboratory by Scheele & Lauffer (1967) who titrated both TMV and TMVP over a wider pH range than Ansevin *et al.*

(1964) had done. They observed that only about 11 of the 15 carboxyl groups present per protein monomer titrated between pH 2.7 and pH 8.0, possibly because the others were involved in salt linkages with positive groups. They confirmed that protons were bound when TMVP polymerised, and deduced an abnormal pK_H around pH 6.5 for the protein.

The presence of non-titratable carboxyl groups was confirmed by Oehlen (1967) who concluded that at least five carboxyl groups were not titrating in the type strain. She also found that only five of the thirteen basic groups could accept a hydrogen ion. However, in a later publication (Paulsen, 1972) she reported that in the virus only eight carboxyl groups of the sixteen were titrating and concluded that only eight were situated near the surface. Of interest in this regard is the report by Budzynski & Means (1971) that only after modification of eight carboxyl groups by amines was aggregation of TMVP at pH 5 completely prevented. This effect appeared not to be solely due to the altered charge on the protein, and it seemed likely that protonation of one or more critically situated carboxyl groups was involved in the polymerisation process. King & Leberman (1973), however, found that only three carboxyl groups of intact TMV (residues 64, 66, and 158) could be modified with cystamine and concluded that these three were thus located towards the outside of the virus particle.

After the demonstration by Durham et al. (1971) that pH was the variable controlling the mode of aggregation of TMVP, titration studies by Butler et al. (1972) indicated that TMV differed from the irreversibly associated stacked-disk rods by possessing two independently titrating groups with pK_H values of 7.1. As the disk had been shown by Butler & Klug (1971) to be the sole structure able to combine with RNA to initiate a nucleoprotein helix, they postulated that the abnormally titrating carboxyls prevented TMVP, under physiological conditions, from forming stable helical rods which would then be unable to coat RNA and form virus rods.

Further support for this hypothesis of the Cambridge group was obtained by Butler & Durham (1972), who found that five TMV variants each possessed two abnormally titrating groups per subunit (see section A2(b) below).

The titration behaviour of TMVP between pH 5 and pH 7 was re-examined by Scheele & Schuster (1975), who reported a reproducible hysteresis in proton binding (i.e. the forward titration curve differed significantly from the reverse curve). By combining titrations with sedimentation velocity analyses, they demonstrated that the reverse titration curve (i.e. using acid titrant) represented the equilibrium state. The hysteresis, which reached a maximum of 0.5 protons per subunit near pH 6.2, was due to the formation at acid pH of metastable helical rods which then depolymerised very slowly as the pH was raised. Their rate of decay decreased from days to minutes within a tenth of a pH unit as the pH was raised above a threshold value near pH 6.6. They concluded that the titration data of Butler et al. (1972) corresponded to the non-equilibrium branch of the titration curve, and suggested that Scheele & Lauffer (1967) failed to observe proton release upon dilution of TMVP simply because the short time scale of their experiments prevented the very slow depolymerisation from becoming apparent.

2. TMV strains and mutants

Various TMV strains and mutants have been compared to the type strain regarding their electrophoretic mobilities (for review, see van Regenmortel, 1972), their acid-base titration properties (Oehlen, 1967; Paulsen, 1972; Butler & Durham, 1972) and the polymerisation characteristics of their proteins (Sarkar, 1960; Rentschler, 1967; Sperling & Klug, 1975).

(a) Electrophoretic comparisons of TMV variants

The electrophoretic mobility is not a reliable method of detecting the amino acid exchanges characterising variants of a particular virus. Thus, Kramer

(1957) found the mobility curves of type strain and dahlemense to be very similar, even though their amino acid sequences differed at about 30 positions, including six differences in basic amino acid locations. The loss or acquisition of charged amino acid residues is not necessarily reflected in a change in electrophoretic mobility. Thus, a TMV mutant having a glutamic acid residue instead of a glycine residue at position 97 could not be differentiated electrophoretically from type strain, although the mobilities of their dissociated proteins did differ (Sengbusch, 1965). In addition, a constant electrophoretic mobility over a given pH range does not mean that residues are not titrating in that range. The mobility of TMV was found to remain constant above pH 5.0 (Kramer & Wittman, 1958), although subsequent titration curves showed a steady increase in base neutralised up to pH 8.0 (Ansevin _et al_., 1964).

(b) Acid-base titration of TMV variants

The titration behaviour of seven TMV variants was compared with that of type strain by Oehlen (1967), who found that between pH 1.8 and pH 6.5 at I=0.1 all the variants, except dahlemense and including U2 and flavum, had titration curves virtually identical to that of type strain. The number of titratable carboxyl groups over that range was identical for all of these. Differences between the titration curves of the strains was observed, however, if they were titrated at a lower ionic strength (I=0.02) from pH 8.0 to pH 4.0, or in 8.4 M urea. Predictably, in the latter solvent each strain was able to bind considerably more protons over that pH range, although surprisingly the extent by which the titration curve of each strain differed from that of type strain could not be correlated with the possession of fewer or more titratable residues.

This study was later extended by the same author (Paulsen, 1972) to include titrations of the proteins of the various strains. The nucleoproteins of each of the eight strains that were titrated contained only eight titratable carboxyl groups. The titration curves of the various mutant proteins were found to differ between pH 6 and 7, which was thought to reflect their differing aggregation behaviour. This was confirmed for the proteins of U2, type strain and dahlemense using opalescence and sedimentation studies. Proton binding commenced above pH 7 for U2, at about pH 7 for vulgare and below pH 7 for dahlemense. The above sequence was the same as that in which their respective proteins were found to aggregate.

Titration studies indicated that the two groups per subunit which titrated with an abnormal pK_H of about 7 in the type strain were also found in the dahlemense, U2 and HR strains of TMV, as well as in the flavum and Ni 109 mutants (Butler & Durham, 1972). These authors compared the titration curves of the nucleoproteins, as well as the proteins, of the various strains with the titration curve of the stacked-disk rod of vulgare protein. For each strain, the differences observed were similar to that found when the titration curve of vulgare protein or nucleoprotein was compared with that of vulgare stacked-disk rods (Butler et al., 1972). This analysis led the authors to propose that the abnormally titrating groups controlled TMV protein aggregation. All the variants titrated were thus shown to have the same mechanism controlling protein aggregation, and the conservation of these groups suggested that their possession was a crucial evolutionary feature. The amino acid pairs involved could be narrowed down to residues 115 and 116, and residue 145 and one other.

(c) Aggregation properties of variant TMV proteins

The aggregation properties of the protein of various TMV strains was compared by Sarkar (1960). He found that the subunits of dahlemense and flavum, but not HR, were able to co-aggregate with subunits of vulgare to form aggregates with electrophoretic mobilities intermediate between that of each alone, indicating that these proteins probably had very similar three-dimensional structures and bonding properties in spite of the differences in their amino acid sequences. The effect of pH and ionic strength on the aggregation of the proteins of vulgare, dahlemense, flavum, HR and U2 was studied by means of sedimentation behaviour by Rentschler (1967). For all the variants, raising the ionic strength to I=0.1 caused aggregation to commence at a higher pH than at I=0.02. Also, protein aggregation was influenced in a similar manner for all the variants by lowering the pH. At I=0.1, protein aggregates sedimenting at about 6 S (i.e. A-protein) were observed at pH 8.0 for all five strains. At pH 7.0, 20-100 S components were present in addition to those of 6 S in the case of vulgare, dahlemense and HR proteins. At pH 6.0 and below, only components of 100 S or more were observed. At pH 7.0 U2 protein appeared to aggregate more slowly than vulgare protein, and dahlemense protein more readily, which was the reverse of the sequence reported by Paulsen (1972). Also, the 20 S particles of dahlemense protein were stable over a wide range of pH values.

Rentschler (1967) also investigated the ability of the proteins of vulgare and dahlemense to co-aggregate with U2 protein. She utilised the fact that aggregated U2 protein migrates electrophoretically more slowly than either aggregated vulgare or dahlemense proteins. Although she found that both U2-vulgare and U2-dahlemense protein co-aggregates formed a single migrating zone of protein in each case, each co-aggregate had an electrophoretic mobility similar to that of the non-U2 protein aggregate alone, and not the intermediate mobility that would have been expected of a mixed aggregate.

A systematic study of the states of aggregation of dahlemense protein was made by Sperling & Klug (1975). They found that dahlemense protein formed similar aggregates to those formed by vulgare protein, but that the conditions for their formation were different. In contrast to vulgare protein, dahlemense protein precipitated when dialysed directly from pH 8 to pH 5. However, if an intermediate dialysis step at pH 7 was included, protein helices were formed at pH 5 presumably due to the formation at pH 7 of 20 S disks essential for the nucleation of helix growth. Also, at neutral or alkaline pH, dahlemense protein did not form long stacked-disk rods like vulgare protein (except in the case of bacterial contamination). If the ionic strength was raised at pH 8, 7 S dahlemense protein first formed 20 S disks, then at $I>1.0$ a 30 S "figure-of-eight" structure consisting of two interlocked disks appeared, and not the short stacks of disks typical of vulgare protein. At $I>1.4$, helical rods appeared, a feature not observed at pH 8 with vulgare protein (Durham, 1972a). Dahlemense protein did not form the short stacks of disks typical of vulgare protein, presumably because of the strong pairing interaction found in the dahlemense protein disk (Sperling _et al._, 1975).

3. Other viruses stabilised by divalent cations

A considerable number of reports exist in the published literature in which divalent cations have been found to either stabilise plant viruses other than TMV, retard their degradation by a variety of agents, or promote assembly or reconstitution of the virion from the protein and nucleic acid components. There are also numerous reports of divalent cations stabilising animal or bacterial viruses, or being required for their _in vivo_ assembly.

(a) The bromoviruses

This group consists of three, small spherical plant viruses which, after TMV, have the best understood assembly and structural properties (Bancroft 1970b; Lane, 1974). These are brome mosaic virus (BMV), broad bean mottle virus (BBMV) and cowpea chlorotic mottle virus (CCMV), which all have diameters of about 25 nm and particle weights of about 5 million daltons.

BMV was shown by Incardona & Kaesberg (1964) to undergo a reversible structural transition and become sensitive to ribonuclease when the pH was raised from 6 to 7. The particle weight remained constant, but the particle radius increased from 138 Å to 155 Å and the sedimentation constant dropped from

87 S to 79 S. Hydrogen ion titration curves of BMV indicated that about two protons per subunit titrated anomalously at pH 6.6, which phenomenon appeared to be related to the structural transition. This reversible rearrangement, which was also demonstrated for CCMV (Bancroft _et al._, 1967), was found to be partially inhibited by the presence of Mg^{2+} (Flanegan & Incardona, quoted in Incardona _et al._, 1973; Bancroft, 1970b). Incardona _et al._ (1973) reported that the transition was a two-step process consisting of a temperature-independent, reversible pH-induced transition, and an irreversible thermal expansion step which could be eliminated by the presence of Mg^{2+}. Dissociation of reassembled BMV capsids as a function of pH near pH 5.5 was not influenced by Mg^{2+}, nor were the hydrodynamic properties of the capsid temperature dependent. These observations led Incardona _et al._ (1973) to suggest that abnormally titrating carboxyls involved in protein-protein interactions were responsible for the pH-induced swelling, while the thermal contribution was due to a change in the conformation of the RNA. These results, suggesting that the viral RNA was the site of Mg^{2+} interaction, were thus in conflict with the earlier assumption of Bancroft (1970b) that the Mg^{2+} ion chelated with two carboxyl groups to position the pair during assembly.

Magnesium and calcium ions were first reported to stabilise BMV, especially at high pH, by Brakke (1963). Using both retention of infectivity and morphological stability as criteria, BMV was stabilised by as little as 10^{-4} M Mg^{2+} at pH 7, and destabilised by EDTA. Later Brakke (1971) found that, whereas BMV treated with bentonite in buffer at pH 9.0 degraded completely to free RNA, addition of Mg^{2+} partly prevented this degradation. Those virions remaining intact could subsequently be degraded with bentonite after chelating the Mg^{2+} with EDTA. Magnesium ions were shown by Pfeiffer & Hirth (1975) to protect the expanded form of BMV against degradation by trypsin, chymotrypsin and ribonuclease at pH 7.4. The thermal expansion step thus exposed the RNA to RNase and unmasked the trypsin and chymotrypsin sites, and was accompanied by the RNA loops extending further from the centre of the particle than in the partially expanded, Mg^{2+}-stabilised BMV (Jacrot, quoted in Pfeiffer & Hirth, 1975).

CCMV behaved similarly to BMV, firstly in that the virion swelled if the pH was raised to near pH 7, which swelling was modified by the presence of Mg^{2+} ions (Bancroft et al., 1967; Adolph, 1975b), and also in that the purified protein was able to aggregate below pH 5.5 to form shells indistinguishable from CCMV capsids (Bancroft et al., 1969; Adolph & Butler, 1974). The precise role of Mg^{2+} ions in reducing the swelling of the CCMV virions is not understood. Although the structure of isolated RNA could be stabilised but not altered by Mg^{2+} (Adolph, 1975a; Jacrot, 1975), Adolph (1975a) found that the structural transition in the virion near neutrality was not due to a change in RNA structure. Jacrot (1975) found that, whereas Mg^{2+} influenced the potentiometric titration curve of the virus, it had no effect on the titration curve of the protein. He concluded, in support of Incardona et al. (1973), that the action of the Mg^{2+} was thus on the RNA or, more likely, on the RNA-protein interaction, and suggested that Mg^{2+} might even break the protein-nucleic acid linkages.

On the other hand, Johnson et al. (1973), who titrated virus but not protein in the presence of Mg^{2+}, found that Mg^{2+} could displace protons from the unassembled protein. They concluded that these protons were not involved in intersubunit linkages and were situated near the protein surface normally exposed on the assembled virus. The site of magnesium action was thought to be a pair of juxtaposed, hydrogen-bonded carboxylic acid groups. The argument in favour of the magnesium reacting with the protein was extended by Bancroft et al. (1973), and supported by Verduin (1974). However, in a recent publication (Bancroft et al, 1976) Bancroft and his co-workers appear to support Jacrot (1975) in that the site of Mg^{2+} action is a protein-RNA linkage. This idea has also been supported, in the case of BMV, by Pfeiffer & Durham (1977).

Titration of CCMV by Johnson et al. (1973) indicated the presence of a hysteresis loop at the pH where the structural transition occurs. The presence of Mg^{2+} abolished this hysteresis, an effect which was explained as being due to the Mg^{2+} ions reducing the repulsion between subunits (probably between adjacent carboxyl groups), thereby preventing the swelling of the virion and thus the exposure of certain basic amino acids responsible for the hysteresis. Jacrot (1975) titrated CCMV and CCMV protein, with results completely different to those of Johnson et al. (1973), which Jacrot explained as possibly being due to CO_2 pick-up in the experiments of Johnson et al. Jacrot found that both the swelling of the virus and the formation of the capsid were accompanied by a titration hysteresis, but that only the virus hysteresis was sensitive to Mg^{2+} ions. He deduced that two carboxyl groups per protein subunit, having raised pK_H values, were responsible for both hysteresis loops. However, in view of the CCMV protein titration curve being unaffected by Mg^{2+}, his results could be interpreted as being due to capsid formation in the absence of RNA slightly raising the pK_H of several carboxyl groups. The RNA in the virus, however, could create two groups with a pK_H near 6.5, which could also participate in the binding of divalent cations and the control of virus disassembly.

(b) Turnip crinkle virus

Turnip crinkle virus (TCV) is a small, spherical virus with a diameter of about 30 nm and a particle weight of about 9 million daltons. It contains 17% RNA (Symons et al., 1963), and 180 protein subunits of molecular weight 38 000, and is structurally similar to tomato bushy stunt virus (Ziegler et al., 1974). Durham (1971) followed breakdown of the virion under various conditions by monitoring the rate of decrease of turbidity. He observed that the rate of breakdown increased sharply as the pH was raised above pH 7.8, which suggested that a group titrating near neutrality was implicated in virus breakdown. The rate of breakdown also increased with ionic strength above I=0.4, indicating that RNA-protein bonding was involved in maintaining the structure of the virion.

(c) Turnip yellow mosaic virus

Turnip yellow mosaic virus (TYMV) is a well-characterised, spherical plant virus (for review, see Matthews & Ralph, 1966) which contains 34% RNA, 180 protein subunits of molecular weight 20 000 daltons, and a large amount of tightly-bound polyamine which neutralises the negative charges on the RNA (Johnson & Markham, 1962). In addition, TYMV binds substantial amounts of divalent cations which appear to resist removal by EDTA (Johnson, 1964).

TYMV is stabilised predominantly by protein-protein bonds (Kaper, 1971). Stable capsids are formed as a by-product of TYMV infection, and comprise a sizeable portion of purified TYMV preparations (Markham & Smith, 1949). Also, the TYMV capsid does not collapse when RNA is released (Kurtz-Fritsch & Hirth, 1972). Only after perturbation of the capsid of TYMV with heat, freezing and thawing, or 12 M formamide, was Kaper (1971) able to observe that TYMV remained stable under mildly acid conditions, but dissociated into capsids and RNA at pH 7 or higher. He concluded that the RNA-protein linkages were dependent on pH, and were broken as the pH was raised to near 7. The stability of the capsid normally precluded detection of the breakage of the RNA-protein bonds. These perturbations of the capsid also unmasked a number of other titrating groups in TYMV.

(d) Other plant viruses

Apart from the above three cases, where the role of cation binding, or of groups titrating near neutrality, in maintaining particle integrity has been actively investigated, there are a number of disconnected reports of virus structure or stability being influenced by divalent cations.

Alfafa mosaic virus (Bos & Jaspers, 1971) consists of bacilliform particles of different lengths, which undergo a reversible conformational change above pH 7.5. This leads to partial separation of RNA and protein, and can be prevented by $MgCl_2$ or positively charged proteins (Verhagen & Bol, 1972).

Tomato aspermy virus (Hollings & Stone, 1971) becomes sensitive to ribonuclease above pH 8, but can then be protected by Mg^{2+} (Habili & Francki, 1974). Southern bean mosaic virus (Shepherd, 1971) is able to bind Ca^{2+} and Mg^{2+}. The capsid undergoes a relaxation of these are removed, and the sedimentation rate drops from 115 S to 100 S. The 100 S virions are sensitive to proteases and RNase (Hsu et al., 1976).

The structures of the filaments of henbane mosaic (Govier & Plumb, 1972), pepper veinal mottle (Brunt & Kenten, 1972) and bean yellow mosaic viruses (Bos, 1970) are affected by Mg^{2+}. The particles are long and straight when exposed to magnesium, but become short and flexuous when exposed to EDTA (Govier & Woods, 1971). Apple chlorotic leaf spot virus (Lister, 1970) was shown by Lister & Hadidi (1971) to have a pH-dependent, structural requirement for divalent cations. Degradation, which occurred only at pH 7 and above, could be prevented by the presence of 1 to 10 mM Mg^{2+}. Two rhabdoviruses, potato yellow dwarf virus (Black, 1970), and wheat striate mosaic virus (Sinha & Behki, 1972) are stabilised by divalent cations (Brakke, 1956; Ahmed et al., 1970).

Cations have also been reported to stabilise animal and bacterial viruses, and to play a role in their correct assembly (see Durham & Hendry, 1977).

B. The binding of divalent cations to proteins

1. Introduction

The binding of metallic cations to proteins is a well-characterised phenomenon, and has been reviewed by Gurd & Wilcox (1956), Steinhardt & Beychock (1964), Steinhardt & Reynolds (1969), Vallee & Wacker (1970), Ainscough & Brodie (1976) and (with reference specifically to the binding of calcium) by Kretsinger (1976) and Kretsinger & Nelson (1976). The proteins concerned can be divided into three general classes. In the first, the metal ions occupy a small number of high affinity sites, are essential for the biological function of the protein, and generally follow the protein in stoichiometric amounts through the ultimate steps of purification. These proteins are usually referred to as metalloproteins, of which haemoglobin, alkaline phosphatase and ferritin are typical examples. Most of these proteins have such high affinities for the metal concerned that association constants have not been measured. The second class of proteins does generally not require the metal for its biological activity, the metal being reversibly bound to specific amino acid residues. The association of the metal ion with the protein can, however, stabilise a specific conformation of the protein necessary for its biological function. Most extracellular hydrolytic enzymes bind cations with moderate affinity, e.g. thermolysin, which is stabilised by Ca^{2+}. The third class consists of certain intracellular proteins (e.g. troponin) which are modulated by cations, particularly Ca^{2+}, acting as a messenger. The cation is bound with high affinity and released many times in response to varying concentrations of cation.

2. General theoretical considerations

(a) Coordinate bonding

Metallic cations are complexed by proteins due to the cations possessing secondary valences able to form coordinate bonds. Each valence has a specific three-dimensional orientation and the number of valences determines the characteristic coordination number of the metal.

Each coordinate bond results from the ability of the metal to act as the acceptor of an electron pair from a donor, or ligand (see e.g. Basolo & Johnson, 1964).

Thus:

Thus:	X	+	:Y	$\longrightarrow$	X:Y
	acceptor (metal)		donor (ligand)		coordination compound

When two or more donor atoms from the same ligand molecule are coordinately bonded to the same metal ion, a chelate complex results. From the above, it follows that the acceptor qualifies as a Lewis acid, and the donor as a Lewis base. This concept thus classifies metal ions as acids, and ligands as bases.

(b) Hydration of the metal ion

The oxygen on a water molecule possesses a nonbonded electron pair, with the result that water can act as a ligand. Thus, in aqueous media, the coordination positions of a metal ion not bonded to a ligand are complexed to water molecules. Such hydrated metal ions are acidic, because the liganded water can lose a proton at an appropriate pH, and a metal hydroxide (often insoluble) results (Williams, 1971a).

The formation of complexes in aqueous solution can thus be regarded as the displacement of water molecules by the ligand in question, i.e.

$$M(H_2O)_x \quad + \quad L(H_2O)_y \longrightarrow ML(H_2O)_z \quad + \quad (x + y - z)H_2O.$$

As far as hydration is concerned, metal ions can be roughly divided into three groups (Gurd & Wilcox, 1956). The first group of metals, when hydrated, lose protons only above pH 11 (i.e. $pK_H > 11$) and hydroxide precipitation begins only at pH>9. Hydrolysis of these metals need not be considered except at high pH. Examples of this group are Ca^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+}. Magnesium is more strongly hydrated than calcium and has a larger hydrated radius (Williams, 1970, 1971b). Members of the second group have pK_H's of between 8 and 11, and certain of their hydroxides are precipitated between pH 6 and 9. Above pH 6 hydrolysis must thus be taken into account when working with these metals, examples of which are Mn^{2+}, Fe^{2+}, Zn^{2+}, La^{3+} and Pb^{2+}. Metals of the third group hydrolyse below pH 7, and the free cations can be studied only at very low pH values. Examples are Hg^{2+}, Cr^{3+} and Al^{3+}.

(c) Stability of coordination compounds

It is known that each metallic cation forms complexes of greater stability with certain ligands than with others. An attempt to explain this phenomenon was made by Pearson (1963) who classified acids and bases as being either "hard" or "soft". Hard acids were of small size, with a high positive charge, and held onto their valence electrons tightly, i.e. were not easily polarisable. Soft acids had a large acceptor atom, with small or zero positive charge, and several valence electrons which were easily removed. Similarly, a soft base was one in which the valence electrons were easily polarised, while a hard base tended to hold its valence electrons more tightly. As a general principle, hard acids then preferred to coordinate with hard bases, and soft acids with soft bases. Thus, hard acids such as the alkaline earth metals and the lanthanides formed the most stable bonds with bases such as OH^-, F^- and CH_3COO^-, while soft acids such as Hg^{2+} or the transition metals formed stable bonds with sulphur or nitrogen atoms as ligands. Hard-soft combinations formed coordination compounds of low stability. Generally, metal ions vary more in their complexing abilities towards sulphur and nitrogen donors, while towards oxygen donors these are more constant (Sigel & McCormick, 1970).

3. The cations associated with proteins

(a) General

The multivalent cations which have been reported to be associated with proteins are Ca^{2+}, Mg^{2+}, Mn^{2+} and Co^{3+} (all hard acids), Fe^{2+}, Cu^{2+} and Zn^{2+} (borderline acids), and Cd^{2+} and Mo^{2+} (both soft acids) (Makinen, 1975). Of these, Ca^{2+} and Mg^{2+} are the metals primarily bound to viruses and involved in their stabilisation (see sections A1(c) and A3, this chapter). These are both hard acids and favour the same ligands. Magnesium, in spite of having the smaller ionic radius, tends to be the softer of the two (Williams, 1970). However, Mg^{2+} is bound preferentially by nitrogen bases, the binding site probably containing a phosphate or carboxylate group as well, while in biological systems Ca^{2+} binding sites are generally multi-carboxylate or -phosphate centres and contain no nitrogen bases (Williams, 1970, 1971b). Manganese (Mn^{2+}), which has a very similar chemistry to magnesium and can in fact replace it in many biological systems, has an affinity for nitrogen ligands, while lanthanum (La^{3+}), although trivalent, binds similarly to Ca^{2+} (Williams, 1971b).

The complexity and chemical structure of proteins influence their behaviour as ligands in a number of ways (Williams, 1971a). Amino acid side chain groups (-COOH, -SH, imidazole) outnumber terminal groups and are thus the dominant contributors to cation binding sites. Because of the restrictions placed by the tertiary structure of a protein on the movement of the polypeptide chain, only a limited number of the potential ligand groups will be accessible to the metal ion, or be in the correct configuration to accommodate the coordinate bond geometries of the metal. A metal will thus select a binding site firstly on a hard-soft basis, and also on the basis of the steric suitability of the ligand groups comprising the site. Coordinate positions on the metal not occupied by the ligand groups of the protein will be occupied by water molecules. Finally, competition between metal ions and protons is important, as either a metal or a proton could accept electrons from a donor. Thus, depending on the pK_H of the ligand, bond formation with a metal will result in the displacement of protons from the site (and also, obviously, of water molecules from both the metal and the site). Finally, the approach of the metal ion might be either assisted or resisted by the charge on the protein.

(b) The binding of calcium by proteins

Today it is recognised that calcium plays an important role in many biological systems (for review, see Kretsinger & Nelson, 1976). This role of calcium is, in many cases, mediated through specific calcium-binding proteins, of which about 70 have now been recognised (for review, see Kretsinger, 1976). The determination of protein-bound calcium has been reviewed by Kretsinger & Nelson (1976) and Steinhardt & Reynolds (1969), equilibrium dialysis and sedimentation or filtration techniques being most commonly employed.

The study of calcium binding to small organic molecules has indicated that oxygen is the preferred coordinating ligand. The coordination number is usually eight (occasionally seven), and the Ca^{2+} ion is usually hydrated. Carboxylate and phosphate groups are particularly strong calcium ligands, the oxygen being the coordinating group in each case.

Detailed structural knowledge of calcium binding by proteins has come mainly from the crystallographic study of carp parvalbumin, troponin, staphyllococcal nuclease, concanavalin A, and thermolysin (Kretsinger & Nelson, 1976). A number of general features of calcium binding by proteins can be discerned. In protein complexes, the coordination numbers with calcium are usually six, with water ligands ranging from zero to three. No nitrogen or sulphur ligands appear to be involved in protein-calcium complexes. Oxygen is the preferred coordinating ligands, the majority of which have been found to be the oxygen atoms of carboxylate groups. However, there appears to be no correlation between the affinity for calcium and the number of carboxylate groups involved in the binding site. In all the cases examined, the binding site also contains a protein main chain (i.e. carbonyl group) oxygen atom. The calcium affinities of most extracellular enzymes is low (pK_{Ca} = 3 to 4), while several intracellular Ca^{2+} binding proteins have very high affinities (pK_{Ca} = 5 to 8). However, a study of the five proteins mentioned earlier has revealed no correlation between ligand type (or ligand geometry) and affinity for calcium, although most of the proteins which bind Ca^{2+} strongly are acidic. Also, the basis of the specificity of Ca^{2+} binding, particularly relative to Mg^{2+}, is not understood. Finally, lanthanides are able to displace bound calcium from most proteins.

The picture that thus emerges of calcium binding sites in proteins is of a "cage" consisting of up to 6 ligands, usually comprising one carbonyl oxygen atom and a number of carboxylate oxygen atoms, situated at the vertices of an octahedron. Up to 3 of the octahedral positions can be occupied by water molecules.

(c) Some factors influencing cation binding by proteins

Obviously, a variety of environmental factors such as pH, ionic strength, competing cations, etc., will affect the binding of a metal by a protein. Only two factors, pH and metal cation concentration, will be considered here.

(i) pH

Metal ions and protons compete for the same sites on a protein molecule (Gurd & Wilcox, 1956; Steinhardt & Reynolds, 1969). Thus, in general terms, as the pH is reduced, one would anticipate a decrease in the metal bound to, and an increase in the protonation of, a cation binding site. Likewise, if the free cation concentration was raised at a fixed pH, the metal ions would displace protons from the binding site, resulting in a reduction in the protonation of the site.

The above can be deduced from the following general expression (Williams, 1953). A ligand A, being the conjugate base of the acid HA with dissociation constant K_H, is able to react with metal M. The following equilibria will apply:

$$M + nA \rightleftharpoons MAn$$

and

$$nHA \rightleftharpoons nH + nA$$

Thus

$$k_M = \frac{[MAn]}{[M][A]^n}$$

and $$K_H = \frac{[H]^n [A]^n}{[HA]^n}$$

Combining the two we obtain:

$$k_M . K_H = K' = \frac{[MAn][H]^n}{[M]\ [HA]^n}$$

from which it can be seen that increasing [H] will result in a decrease in [MAn] and an increase in [M] and [HA]. Increasing [M] will cause [HA] to decrease and [MAn] and [H] to increase.

More sophisticated analyses can be found in Gurd & Wilcox (1956), Tanford (1962) and Steinhardt & Reynolds (1969). The last-mentioned authors, for example, showed that if a cation M bound with association constant k to a ligand L having n independent, identical binding sites, then the average number of metal ions bound could be defined as:

$$\nu = \frac{\text{moles combined M}}{\text{total moles L}}$$

$$= \frac{nk[M]}{1 + k\,[M]}$$

which could be arranged to:

$$\frac{\nu}{[M]} = k\ (n-\nu)$$

(This equation can be used to obtain n as $\frac{\nu}{[M]}$ tends to zero in a plot of $\frac{\nu}{[M]}$ vs. ν).

If two cations A and B competed for this set of n sites, each cation with a different association constant, the above equation for ν became:

$$\nu_A = \frac{k_A\,[A]}{1 + k_A\,[A]}\ (n-\nu_B)$$

and $$\nu_B = \frac{k_B\,[B]}{1 + k_B\,[B]}\ (n-\nu_A)$$

which by algebraic combination led to:

$$\nu_A = \frac{nk_A [A]}{1 + k_A [A] + k_B [B]}$$

and $$\nu_B = \frac{nk_B [B]}{1 + k_B [B] + k_A [A]}$$

The above equations were applicable to the competition between metal ions and protons, provided the electrostatic interaction was taken into account. This interaction Steinhardt & Reynolds (1969) showed could be expressed by the equations

$$k'_{metal} = k \exp(-2wZ\ z_{metal})$$

and $$k'_{hydrogen} = \frac{1}{K_H} \exp(-2wZ)$$

where k' was the apparent association constant, k the intrinsic association constant for the metal, Z the net charge on the protein, z_{metal} the charge on each metal ion, K_H the intrinsic dissociation constant for protons, and w was the electrostatic interaction factor which depended on the radii of the protein and ions concerned and on the ionic strength. The equation for competitive binding thus became:

$$\frac{\nu_{metal}}{n-\nu_{metal}} = \frac{k \exp(-2wz_{metal} Z)\ C_{metal}}{1 + a_H+ / (k'_H) \exp(2wZ)}$$

The intrinsic association constant of the metal for the protein could then be calculated, having obtained values of ν and C_{metal} from equilibrium dialysis, and of a_H+, Z, w and k_H from the acid-base titration curve on the protein in the absence of the metal.

The above equation was only applicable to the situation where one proton competed with one metal ion for a single site. The situation became more complex if a number of sites on the protein was involved, each having a different k and K_H, or if one metal cation displaced more than one proton.

(ii) Metal cation concentration

In the case of protein-metal complexes with very high binding energies (see section B1, this chapter), the assumption is made that added metal is bound stoichiometrically to the protein until all the binding sites are filled. This may be correct in practical terms only, as even distilled water may, due to contamination, have an insufficiently low level of the metal to induce dissociation of the metal from the protein by exhaustive dialysis (Steinhardt & Reynolds, 1969). However, if proteins have only moderate affinity for the metal, the association is practically reversible and association constants can be determined experimentally.

The binding of a metal M to a ligand L can be expressed in the ideal case, assuming no binding of protons, by the equation:

$$M + L \rightleftharpoons ML$$

for which we can obtain a dissociation constant

$$K_M = \frac{[M][L]}{[ML]}$$

therefore

$$pK_M = pM - \log \frac{[L]}{[ML]}$$

$$= pM - \log \frac{\alpha}{1-\alpha}$$

where α is the fraction of the total ligand to which no metal has bound. Thus, the binding of a metal to a ligand is completely described by the pK_M, and one requires only the metal concentration to establish the degree of binding. Further, if $\alpha = 0.5$, then the $pK_m = pM$. Thus the pK_M equals the pM value (i.e. $- \log_{10} [M]$) when 50% of the ligand is bound to metal.

The affinity of a protein for calcium is, broadly speaking, a reflection of the calcium concentration of the normal environment of that protein. Thus, an intracellular protein such as troponin, for which calcium has a messenger function, has a pK_{Ca} of about 7, while the calcium concentration of cytoplasm is normally of the order of 10^{-7} M (Kretsinger & Nelson, 1976). Extracellular calcium binding proteins such as thermolysin have pK_{Ca}'s of the order of 3 or 4, which is the approximate pCa of most extracellular fluids.

C. The acid-base titration of proteins

1. Hydrogen ion equilibria

Detailed discussions of hydrogen ion equilibria as applied to proteins can be found in physical biochemistry textbooks such as Bull (1964), Martin (1964), Tanford (1961), or van Holde (1971).

A chemical reaction will reach equilibrium when the free energy, G, of the reactants is equal to the free energy of the products, i.e. when $\Delta G = G_{products} - G_{reactants} = 0$. The free energy of n moles of a substance is related to the effective concentration (or activity, a) by the expression:

$$G - G^{o} = nRT \ln a$$

where G^{o} is the free energy in the standard state where, by definition, a = 1, R is the universal gas constant and T is the absolute temperature. As the concentration of a substance tends to zero, so does the activity tend to equal the molality; activity is thus a reflection of the fact that molecules or ions of a substance in solution do not act independently of each other. Initially, for spontaneous reactions, values of ΔG are negative, and the reaction occurs until $\Delta G = 0$.

It can be shown (see, for example, Martin, 1964) that for a general reaction:

$$aA + bB \rightleftharpoons pP + qQ$$

the value of ΔG, when the concentrations (strictly the activities, but it will be assumed that the solutions behave ideally) of A, B, P, and Q have general values [A], [B], [P] and [Q], is related to ΔG^o, i.e. the free energy change for the reaction under standard conditions with $[A] = [B] = [P] = [Q] = 1 \text{ mol } l^{-1}$, by

$$\Delta G = \Delta G^o + RT \ln \left\{ \frac{[P]^p [Q]^q}{[A]^a [B]^b} \right\}_{\text{general values}}$$

At equilibrium, $\Delta G = 0$ and the concentrations reach their equilibrium values. Hence:

$$\Delta G^o = -RT \ln \left\{ \frac{[P]^p [Q]^q}{[A]^a [B]^b} \right\}_{\text{equilibrium values}}$$

i.e. $\Delta G^o = -RT \ln K$

where K is the equilibrium constant in terms of concentrations.

For the simple reaction $A \rightleftharpoons B$:

$$\Delta G = \Delta G^o + RT \ln \frac{\alpha}{1 - \alpha}$$

where α is the fraction of the total moles of A and B that are products, and $1 - \alpha$ is the fraction of reactants remaining. If the weak acid HA ionises as follows:

$$HA \rightleftharpoons A^- + H^+$$

then $$K_H = \frac{[H^+][A^-]}{HA}$$

and $$\alpha = \frac{[A^-]}{[HA] + [A^-]}$$

therefore $$K_H = \frac{[H^+]\,\alpha}{1 - \alpha}$$

and $$1-\alpha = \frac{[H^+]}{[H^+] + K_H}$$

The free energy change for the ionisation is (Martin, 1964):

$$\Delta G_a = RT \ln(1 - \alpha)$$
$$= 2.3\ RT \log \frac{[H^+]}{[H^+] + K_H}$$

which at 25°C and pH 7 becomes:

$$\Delta G_a = 1.36 \log \frac{10^{-7}}{10^{-7} + K_H} \quad \text{kcal mol}^{-1}$$

In the case of the conjugate base, the equation is:

$$\Delta G_b = 1.36 \log \frac{K_H}{10^{-7} + K_H} \quad \text{kcal mol}^{-1}$$

Thus, knowing the K_H of the reaction, the free energy change for ionisation at any pH can be calculated.

Free energy changes have two contributing terms, i.e.:

$$\Delta G = \Delta H - T\Delta S$$

where ΔH = the change in enthalpy, which is a measure of the conversion of the energy released or absorbed at heat, ΔS = the change in entropy, which is a measure of changes in order at the molecular level, and T = the absolute temperature.

A spontaneous reaction will have a negative free energy change, the more negative the value of ΔG the greater the driving force of the reaction. Thus, for the ionisation of acetic acid at 25°C, $\Delta H^o = -0.1$ kcal mol^{-1} and $\Delta S^o = -22.1$ cal K^{-1} mol^{-1} (Martin, 1964). It therefore follows:

$$\Delta G^o = \Delta H^o - T\Delta S^o$$
$$= -0.1 - 297(-0.0221)$$
$$= +6.46 \text{ kcal mol}^{-1}.$$

The positive value for ΔG^o indicates, as expected, that reaction in the direction $HAc \longrightarrow H^+ + Ac^-$ is not spontaneous under standard conditions, where $[HAc] = [H^+] = [Ac^-] = 1$ mol l^{-1}. Under these conditions, $H^+ + Ac^- \longrightarrow HAc$ is spontaneous. Since:

$$\Delta G^o = -RT \ln K$$

a large positive value of ΔG^o corresponds to a small value of K, i.e. to a small proportion of products to reactants, and thus to limited ionisation of an acid. Normally, if $\Delta G^o > +10$ kcal mol^{-1} products are hardly detectable at equilibrium.

From the van't Hoff equation (see e.g. Bull, 1964):

$$\frac{d \ln K}{dT} = \frac{\Delta H^o}{RT^2}$$

it can be seen that for a large positive value of ΔH^o, the equilibrium constant K and hence the proportion of products at equilibrium, or the extent of ionisation of an acid, increases markedly with increasing temperature. Thus, the small ΔH^o term for acetic acid illustrates the general insensitivity of the pK_H of carboxylic acids to temperature.

For the ionisation of the amino groups on glycine at 25^oC, $\Delta H^o = 10.6$ kcal mol^{-1} and $\Delta S^o = -9.4$ cal K^{-1} mol^{-1}. Thus, $\Delta G^o = +7.8$ kcal mol^{-1}. The large ΔH^o term indicates that heat effects dominate in ammonium ionisations, and that the pH of amino buffers (e.g. tris) are strongly temperature dependent. Water is amphiprotic, i.e. can ionise as a base or as an acid:

$$H_2O + H_2O \rightleftharpoons H_3O^+ + OH^-$$

$$K_w = \frac{[H_3O^+][OH^-]}{[H_2O]^2}$$

$$= [H^+][OH^-] \quad ([H_2O] \text{ is constant})$$

$$= 1 \times 10^{-14}$$

$$= \text{ion product constant for water.}$$

The ionisation of an acid can be expressed as:

$$HA + H_2O \rightleftharpoons H_3O^+ + A^-$$

$$K_H = \frac{[H^+][A^-]}{[HA]} \quad ([H_2O] \text{ is constant})$$

$$pK_H = pH - \log \frac{[A^-]}{[HA]}$$

therefore $$pK_H = pH - \log \frac{\alpha}{1 - \alpha}$$

Thus if α (the fraction ionised of the total amount of acid) is equal to 0.5, then $pK_H = pH$. The pK_H of an acid or base is thus that pH at which 50% of the acid or base exists in the ionised form. It is also obvious that the last equation above permits calculation of the degree of ionisation of a substance at any pH provided the pK_H is known.

2. The general titration behaviour of proteins

Most proteins have, on the average, one titratable group per every three or four amino acids. Each protein thus has a characteristic titration behaviour, the individual features of which have been reviewed by Tanford (1962) and Steinhardt & Reynolds (1969).

The amino acids which contain ionisable groups together with the pK_H of each group as observed in simple model compounds, are listed in Table 1.

TABLE 1

Prototropic groups found in proteins

Amino acid residue	Ionisable group	pK_H [a]	Ref.
C terminal	α-carboxyl	3.4	2
aspartic acid	β-carboxyl	4.08	1
glutamic acid	γ-carboxyl	4.50	1
histidine	imidazole	6.3	2
cysteine	thiol	8.3	2
tyrosine	phenol	9.6	2
lysine	ε-amino	10.4	2
arginine	guanidyl	>12.0	2
N terminal	α-amino	7.5	2

[a] The values given are those expected in proteins, based on pK_H values determined using free amino acids.

1. Nozaki & Tanford, 1967. 2. Steinhardt & Beychock, 1964.

In addition, each protein possesses a N-terminal amino group and a C-terminal carboxyl group, each of which will ionise depending on whether in the intact protein they are exposed to the aqueous environment or not.

The pK_H's of the prototropic groups listed in Table 1 will be influenced by other charged groups present on the protein. These electrostatic effects are relatively long range, and can cause the pK_H of a group on a protein to differ by as much as 1.5 from the pK_H of that group when situated in a model compound. At low pH, the positive charges on a protein will repel protons and pK_H's will tend to be reduced while at higher pH the negative charge will attract protons and thus raise the pK_H's. The net effect is a broadening of the titration curve for any one group relative to that of the monobasic acid. The magnitude of this electrostatic effect decreases with increasing ionic strength, due to the "swamping effect" that ion atmospheres have on charged groups. With increased ionic strength, interactions between charged groups decrease and the titration behaviour of individual sets of like groups become more apparent (see, for example, Martin, 1964).

Certain proteins undergo conformational changes as the charge on the macromolecule is increased. This unfolding can result in formerly buried groups being exposed to the environment, which in turn results in their titration at a pH to some extent removed from the normal pK_H of that group. This mechanism has been invoked to explain the anomalous titration of carboxyl groups following a conformational change in bovine serum albumin (Vijai & Foster, 1967).

In addition, the folding of the polypeptide chain of many proteins buries potentially ionisable residues in the hydrophobic interior of the molecule. These buried residues will thus not be available for titration. Thus, Paulsen (1972) reported that in TMV only eight of the sixteen carboxyl groups in TMV protein were available for titration. There are also buried imidazole groups in haemoglobin (Steinhardt et al., 1962) and phenolic groups in RNase

(Tanford et al., 1955). Similarly, if access of the solvent to the buried residues is restricted, they will titrate at an anomalous pH. Carboxyl, sulfhydryl and phenolic groups often titrate abnormally because they are buried in a region of low dielectric constant, and their ionisation creates two charges where previously none existed. Ammonium ions are less affected by transfer to an apolar region, as new charges are not created or destroyed:

$$-COOH + H_2O \rightleftharpoons -COO^- + H_3O^+$$

$$-NH_3^+ + H_2O \rightleftharpoons -NH_2 + H_3O^+$$

This phenomenon could possibly account for the anomalously titrating thiol group in β-lactoglobulin (Nozaki et al., 1959).

Hydrogen bonding between a titratable residue and one adjacent to it can result in anomalous titration of the former. Thus, it is thought that adjacent carboxyl groups can form a carboxyl-carboxylate pair and that the residual hydrogen-bonded proton then dissociates at an elevated pH. This phenomenon has been reported for TMV (Butler et al., 1972) and for BMV (Incardona & Kaesberg, 1964), and could possibly explain the observed titration at pH 7.3 of two carboxyl groups in β-lactoglobulin (Nozaki et al., 1959). Similarly, it will be more difficult to add a proton to a hydrogen-bonded base.

Involvement of the titratable group in an ionic linkage affects the protonation. Carboxylate groups may be stabilised in the charged form by adjacent amino groups and are then available for protonation only at a pH lower than the normal pK_H, as postulated by Steinhardt & Reynolds (1969) for bovine serum albumin. In certain proteins, groups are rendered non-titratable by very strong complex formation with a metallic cation, as in the case of zinc insulin (Tanford & Epstein, 1954) and *E.coli* alkaline phosphatase (Reynolds & Schlesinger, 1968). Non-titratable charged carboxylate and amino groups have been reported in lysozyme and chymotrypsinogen respectively (Tanford, 1962), for which no satisfactory explanation has been provided.

The net result of the effects mentioned above is that it is generally not possible unambiguously to divide protein titration curves into an acid branch representing the titration of carboxyl groups, a neutral branch representing the titration of imidazole and α-amino groups, and an alkaline branch representing the titration of tyrosyl, ε-amino and guanidyl groups. The phenomenon of anomalously titrating groups in proteins is extensively discussed in the two reviews cited at the start of this section.

3. Interpretation of protein titration curves

(a) Theoretical construction of titration curves

The titration curve of a protein of known amino acid composition could be deduced from theoretical considerations alone, provided all the prototropic groups present titrated and provided there was no electrostatic interaction between these groups. As neither of the above two provisos holds in the case of proteins, attempts have been made to evaluate the electrostatic effects operative in proteins (Hill, 1955; Tanford & Kirkwood, 1957; Tanford, 1957). To date, however, no satisfactory account of a protein titration curve has been derived from theoretical principles alone. K. Linderström-Lang in 1924, by extending to proteins the earlier work of P. Debye and E. Hückel in 1923, deduced that the following equation applied to globular macromolecules:

$$pH = pK_{int} + \log \frac{\alpha}{1-\alpha} - 0.868wZ$$

where Z = mean net charge on the protein

and w = electrostatic interaction factor (which in water at 25°C depended only on the radius of the particle and the ionic strength).

Expressed in other terms, the intrinsic ionisation constant of a set of groups (pK_{int}) was modified by the presence of charge according to:

$$pK = pK_{int} - 0.868wZ$$

The above equation is often used to construct protein titration curves. However, Linderström-Lang made the assumptions that the particle was spherical with constant radius, that all the charges were uniformly distributed near the surface with no buried groups, and that all the charged groups were in environments of identical dielectric constant. These assumptions are in a large measure responsible for the discrepancies found between calculated and observed titration curves. In addition, if unfolding, swelling, denaturation or aggregation of the protein occurs during a titration, deviations from the Linderström-Lang model can result (Tanford, 1962). The anomalous titration behaviour discussed in the preceeding sub-section is obviously also significant in this regard.

(b) Practical titration reference points

The practical interpretation of protein titration curves is discussed fully by Tanford (1962), who describes a titration curve as a record of the number of protons attached to a protein molecule at any pH relative to the number attached at an arbitrary reference pH. Three points on a titration curve have actual physical significance, however. These are:

(i) The acid end point, or point of maximum proton charge. At this pH, all the titratable groups will be in their protonated form, and the addition of more acid will result in no further proton binding. Some proteins denature before the acid end point is reached e.g. haemoglobin (Steinhardt & Zaiser, 1951), while with TMV only an approximate acid end point can be determined (Scheele & Lauffer, 1967).

(ii) The point of zero net proton charge. A protein solution can be freed of all small exchangeable ions except H^+ and OH^- by passage through a mixed-bed ion-exchange resin. The protein solution is then isoionic and the protein molecules have a charge negligibly different from zero. The isoionic point is defined theoretically as that pH at which the number of positive and negative groups arising exclusively from proton exchange are equal to each other, and is defined practically (provided the protein concentration is at least 1% and the isoionic point is not too far removed from pH 7) as the pH of a solution of isoionic material.

Titrations are customarily performed in the presence of a neutral salt, ions of which will bind to the protein and thus alter the charge of the originally isoionic macromolecules. The binding of these ions will only marginally affect the binding of protons, and the net proton charge (the molecular charge due to bound protons) will remain close to zero. Provided the isoionic point falls between pH 5 and pH 9 and the protein concentration is more than 1%, the isoionic point is practically equivalent to the point of zero net proton charge; the latter can be calculated exactly from the pH of the solution (see Tanford, 1962).

(iii) The point of minimum proton charge. The alkaline end point is usually more difficult to define experimentally than is the acid end point. Irreversible degradation usually sets in as pH 12 is approached. The alkaline end point is thus that pH where all the groups (except perhaps guanidyl) have been converted to their basic form.

(c) The counting of groups

With due regard to the previously discussed effects of electrostatic interactions, of groups being buried in apolar environments, and of hydrogen-bonding, various regions of a protein titration curve can tentatively be ascribed to particular groups (Tanford, 1962). As mentioned in Section 2 of this chapter, the separation between the titration of these groups is more pronounced if the ionic strength $I \geq 0.1$. Thus, the acid portion of a titration curve can tentatively be assigned to carboxyl groups, which should all be in their basic form by pH 6. From Table 1, it can be seen that no other groups have pK_H's within two pK_H units of the carboxyl groups. The neutral region of the curve can be assigned to imidazole and α-amino groups, while the alkaline region can be taken to represent ε-amino, phenolic and possibly sulfhydryl groups. Phenolic groups can be titrated separately by a spectrophotometric method (Martin et al., 1958) and subtraction of this number from the total number of groups titrating in the alkaline region yields the number of side-chain amino groups.

At the acid end point, only the basic groups (amino, imidazole and guanidyl) will bear charges, which will all be positive. The number of protons required to go from the point of zero net proton charge to the acid end point will thus be equivalent to the sum of these groups. If the number of amino and imidazole groups have been obtained by counting, the number of guanidyl groups can be calculated.

An extension of the group counting procedure is the detection by titration of a conformational change in a protein following a particular treatment (Tanford, 1962). Differences in the titration curves of a protein in different solvents, or before and after combination with, for example, a metal, yield information about the exposure of previously buried groups, or groups having altered pK_H's due to certain residues being involved in the binding of a substance. From the titration curves obtained before and after the treatment, a difference curve can be calculated which can be one of three types (Tanford, 1962). Firstly, new titratable groups can appear, due to the treatment unfolding the protein and exposing prototropic groups, e.g. in the case of β-lactoglobulin (Nozaki et al., 1959). Secondly, no new groups appear, but the pK_H of certain groups is altered by the treatment. This is the situation obtained if an ionisable group is involved in complexing a metal, e.g. the binding of zinc by carboxypeptidase (Coleman & Vallee, 1961). Finally, the number of groups counted in each class does not change with the treatment, but the titration curve has an altered shape.

Difference curves can be obtained either by comparing complete titration curves obtained before and after treatment, or by using a pH-stat (Jacobsen et al., 1957) which maintains a constant pH by the addition of acid or alkali. The total amount of titrant required is thus a measure of the difference at that pH between the corresponding titration curves. The pH-stat method is, however, the less informative of the two, especially if the titration curves alter with time (i.e. show time dependence) or are not reversible (i.e. show hysteresis), both of which phenomena indicate that the protein is undergoing either denaturation or polymerisation (Tanford, 1962).

4. Hydrogen ion titration as an indicator of metal binding by proteins

Protons and metallic cations can compete for the same ligands in proteins (see this chapter, section B3(c)). This ability of metallic cations to displace protons from ligands has meant that hydrogen ion titration can be used as a method of studying metal-ligand binding (Gurd & Wilcox, 1956). General equations for this competition are discussed in section B3(c).

Two titrational approaches have been employed. The one consists of monitoring the pH shifts produced by the addition of small amounts of cation to a solution of ligand or protein. This method was used, for example, by Coleman & Vallee (1961) to follow the binding of Zn^{2+} to carboxypeptidase A. Metal was added to apoenzyme at a variety of pH values, and a pH stat was employed to measure the amount of base required to maintain a constant pH. This yielded a plot, essentially a difference-titration curve between native enzyme and apoenzyme, with points of inflection at pH 7.7 and pH 9.1. These pH values corresponded to the pK_H's of an α-amino and a thiol group respectively, to which Zn^{2+} was apparently chelated. Tanford (1976) has, however, claimed that this latter interpretation is an oversimplification, on the grounds that the curves of Coleman & Vallee (1961) are too steep to represent plots of the type normally obtained from the equation:

$$pK_H = pH - \log \frac{\alpha}{1-\alpha}$$

(see section C1 of this chapter). Breslow & Girotti (1966) used a similar approach to study the binding of Cu^{2+} to ribonuclease.

The second titrational approach is to titrate the ligand or protein first in the absence and then in the presence of a small amount of the cation. Comparison of the two curves yields a difference-titration curve which is completely analogous to that obtained using the first approach mentioned above. Imidazole was titrated in the absence and in the presence of both Zn^{2+} and Cu^{2+} by Edsall _et al_. (1954), and Tanford & Epstein (1954) used a similar approach to study the binding of Zn^{2+} to insulin. The latter authors presented evidence that Zn^{2+} was bound to two imidazole groups per insulin molecule. Using spectrophotometric titration, Wishnia _et al_. (1961) demonstrated a difference of six titratable phenol groups between conalbumin and its iron complex. They concluded that each of the two iron (III) ions bound per protein molecule was thus chelated to three phenolate residues.

An examination of the published literature reveals, however, that this technique has not been extensively employed to study protein binding of cations, particularly of calcium. This is certainly mainly due to the complexities involved in interpreting situations where a cation competes with protons for more than one class of sites on a protein, each with a characteristic association constant for both the metal and hydrogen ions. A discussion of this problem, as well as of the mathematical treatments applicable to simpler situations, can be found in Gurd & Wilcox (1956), Tanford (1962) and Steinhardt & Reynolds (1969).

D. Experimental objectives of the present study

The evidence that bound divalent cations, particularly calcium and magnesium, have a stabilising function in many viruses has been presented in section A of this chapter. The further postulate of Durham & Butler (1975), that dissociation of calcium ions from specific binding sites on the TMV particle could act to trigger intracellular disassembly, leads to a number of _prima facie_ predictions that are experimentally verifiable:

(1) _In vitro_ removal of bound cations from TMV under approximately physiological conditions should result in measurable dissociation of protein from the virion.

Brakke & van Pelt (1969) reported that TMV does not degrade at pH 7.0 or pH 7.5 in buffers containing 1 mM EDTA. In addition, they reported a personal communication from R.L. Steere that a TMV preparation had remained monodisperse for over a year in 1 mM EDTA at pH 7.5. On the other hand, dissociation of TMV at pH 9 has been found by Shalaby & Lauffer (1967) to be favoured by the presence of EDTA.

It was reasoned that by selection of the appropriate virus concentration, pH, ionic strength and EDTA concentration, a comparable effect of EDTA should be demonstrable under quasi-physiological conditions (i.e. pH $\simeq$ 7, I $\simeq$ 0.1) in spite of indications of Brakke & van Pelt (1969) to the contrary. This was investigated by dialysing TMV preparations of different concentrations against solutions of various EDTA concentration, pH, and ionic strength and then assaying the supernatant for protein after removal of intact virus particles by centrifugation.

(2) Tobacco mosaic virus should bind measurable amounts of calcium, with a dissociation constant of the order of 10^{-5} M, and should have a greater affinity for calcium than for other divalent cations, particularly magnesium.

For calcium to function as a stimulus for TMV dissociation, the TMV particle must possess sites able to bind calcium ions. These sites should have a sufficiently strong affinity for calcium to ensure that in most extracellular environments, where the calcium concentration is $\geq 10^{-5}$M, calcium would be bound and the particle would be stabilised. Since the calcium concentration in cytoplasm is of the order of 10^{-6}M to 10^{-7}M (Durham, 1974; Kretsinger & Nelson, 1976), one could expect calcium ions to dissociate in the cell, thereby triggering disassembly of viruses. The above conditions require the binding sites to have a pK_{Ca} in the region of 5. Further, magnesium concentration in cytoplasm is generally higher than that of calcium (Williams, 1971a). The calcium binding sites should thus have a significantly lower affinity for magnesium to prevent the binding of Mg^{2+} ions to TMV _in vivo_ from counteracting the trigger effect of calcium dissociation.

Three techniques were employed to investigate divalent cation binding to TMV: (1) equilibrium dialysis of TMV against suitable buffers containing known amounts of radioactive calcium;

(2) a centrifugal technique in which the amount of bound radioactive calcium sedimenting with the virus was determined; and

(3) potentiometric titration of TMV in the absence and presence of known amounts of divalent cation. The applicability of the last-mentioned technique follows from the assumption that carboxyl groups with raised pK_H values are involved in the binding of divalent cations (Durham & Butler, 1975), which would alter the hydrogen-ion titration behaviour of TMV.

(3) The affinity for calcium of TMVP could be significantly lower than that of TMV.

If the dissociation of calcium ions from TMV results in the liberation of protein subunits from the intact virion, it is reasonable to assume that calcium will bind more weakly to TMVP than to TMV. This prediction is supported by the indication that the calcium tightly bound to TMV is complexed via a site involving the RNA (see section Al(c) of this chapter). Shalaby _et al_. (1968) reported that TMVP bounds negligible amounts of calcium, except below pH 4, when two Ca^{2+} ions per subunit are bound. On the other hand, in the same laboratory McMichael & Lauffer (1975) reported that TMVP was able to bind up to two Ca^{2+} ions per subunit at pH 5.5.

Calcium binding to TMVP was investigated in the present study by equilibrium dialysis and by hydrogen-ion titration.

(4) Other TMV strains, as well as other plant viruses, should possess calcium binding sites.

The proposed role of calcium ions in TMV disassembly should be a phenomenon not restricted to strain vulgare, but should apply to other strains of TMV as well. Thus, other strains of TMV should possess calcium binding sites and their calcium binding properties should resemble those of vulgare to a greater or lesser extent. By comparing the cation-binding affinities of the type, Y-TAMV, U2 and cowpea strains of TMV with their amino acid sequences (Hennig & Wittmann, 1972), it may prove possible to localise the binding sites in these strains. These results can be interpreted in the light of the predicted folding of the polypeptide chain of vulgare (Durham & Butler, 1975), certain features of which have been confirmed by X-ray diffraction (Holmes _et al_., 1975).

In the light of certain spherical plant viruses having been reported to either contain or be stabilised by divalent cations (e.g. Lane, 1974; Johnson, 1964; Verhagen & Bol, 1972; Habili & Francki, 1974; Hsu _et al_., 1976) the possession of cation binding sites should be demonstrable for other plant viruses as well.

Brome mosaic virus (BMV) exhibits both a titration hysteresis, and a capsid swelling near neutrality that is influenced by divalent cations.

pH 6.5. The titration behaviour of TMVP has been examined in a number of laboratories and is known from the work of Scheele & Lauffer (1967), Butler et al. (1972) and Scheele & Schuster (1975) to reflect the polymerisation processes occurring at the different pH's. As titration hysteresis of TMVP indicates a change in polymerisation state (Scheele & Schuster, 1975), a calcium-induced alteration of TMVP's titration hysteresis would indicate an effect of calcium on TMVP polymerisation.

The aggregation behaviour of the proteins of other strains of TMV should similarly be affected by Ca^{2+} ions. These results can be correlated with the aggregation (Durham, 1972a) and titration (Scheele & Schuster, 1975) behaviour of the protein of TMV vulgare. While there is evidence, obtained primarily from co-aggregation (Sarkar, 1960; Rentschler, 1967) and X-ray diffraction (Holmes et al., 1975) studies, that the proteins of the different TMV strains have fundamentally similar structures, there is also evidence that they do not all show an identical polymerisation behaviour to that of vulgare protein (Sperling & Klug, 1975).

During the titrations of the proteins of the four TMV strains, it became apparent that neither their aggregation behaviour nor their response to calcium ions could be adequately elucidated by hydrogen-ion titration alone. The protein aggregates that formed at different pH values, in both the absence and presence of calcium ions, were therefore examined by analytical ultracentrifugation.

In summary, it was hoped that by combining the techniques of equilibrium dialysis, sedimentation and hydrogen-ion titration it would be possible to either verify or refute the above five predictions that follow from the postulate that calcium ions act as an intracellular trigger for viral uncoating. Attempts were also made to obtain an indication of the extent, nature and possible alternative functions of divalent cation binding by various TMV strains as well as their proteins and a number of other plant viruses.

CHAPTER THREE

MATERIALS AND METHODS

A. Viruses

1. Virus strains and their origins

The following tobacco mosaic virus (TMV) strains were used in this study: vulgare (Zaitlin & Israel, 1975); Y-TAMV (Knight *et al.*, 1962); U2 (all obtained from Dr C.A. Knight, Virus Laboratory, Berkeley, California, U.S.A.); and cowpea strain, also known as sunn-hemp mosaic virus (Kassanis & Varma, 1975), obtained from Mr M.W. Rees, John Innes Institute, Norwich, England. The strain identities were confirmed by amino acid analysis of their purified proteins, for which gratitude is expressed to Mr G. Rodrigues, Biochemistry Department, University of Cape Town (see Results, section A1).

The brome mosaic virus (BMV) (Bancroft, 1970a) used was a culture originally isolated in South Africa from wheat, *Triticum aestivum* L. (von Wechmar & van Regenmortel, 1966). Turnip crinkle virus (TCV) (Hollings & Stone, 1972) was obtained from Dr P.J.G. Butler, Cambridge, England, while turnip yellow mosaic virus (TYMV) (Matthews, 1970), Cambridge strain, was obtained from Dr R. Markham, John Innes Institute, Norwich, England.

2. Virus purification

All virus preparations used in this study were provided in a semi-purified form by Dr M.B. von Wechmar, Department of Microbiology, University of Cape Town.

TMV strains were purified by the procedure of von Wechmar & van Regenmortel (1970). Leaves of infected tobacco plants (*Nicotiana tabacum* L. cv. Turkish), or of broad bean plants (*Vicia faba* L.) in the case of the cowpea strain, were homogenised in sodium ethylenediamine tetraacetate (EDTA) pH 9.5 and, after centrifugation to remove particulate matter, the juice was further clarified by filtration through a pad of activated charcoal and diatomaceous earth. The virus was precipitated from the opalescent filtrate by the addition of polyethylene glycol (PEG), resuspended in phosphate buffer, and further purified by differential ultracentrifugation.

BMV was purified by the procedure of von Wechmar & van Regenmortel (1966). Leaves of infected wheat plants (Triticum aestivum L.) were homogenised in a Waring blender in phosphate buffer pH 7 containing chloroform, and the sap was clarified by acidification to pH 4.0 with 10% acetic acid, followed by centrifugation at 8 000 g for 10 min. The virus was further purified by differential ultracentrifugation and maintained in pH 4.4 buffer.

TYMV was purified by homogenising leaves of infected chinese cabbage plants (Brassica pekinensis cv. Wong Bok) in 0.1 M acetate buffer pH 4.8, adjusting the pH of the slurry to 4 with 10% acetic acid, and clarifying by centrifugation at 8 000 g for 10 min. Ammonium sulphate was added to a final concentration of 45% (weight/volume) and, after standing overnight, the precipitate which formed was centrifuged off at 8 000 g for 10 min and resuspended in 0.05 M acetate buffer pH 4.8. The virus was further purified by differential ultracentrifugation (Matthews, 1960).

TCV was purified from chinese cabbage (Brassica pekinensis cv. Wong Bok) by a modification of the method of Leberman (1966). Infected leaves were homogenised in acetate buffer pH 5.0, the juice was clarified by centrifugation at 8 000 g for 10 min, and the virus precipitated by the addition of PEG. The pellets were resuspended in pH 5.0 acetate buffer and further purified by differential ultracentrifugation.

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3. Preparation of TMV protein

The proteins of the various TMV strains were prepared by the method of Durham (1972a). Approximately 50 ml of purified virus at a concentration of 5 to 10 mg/ml were dialysed overnight at $4^{o}C$ against 2 l of 0.1 M ethanolamine adjusted to pH 11 with N HCl, during which time the opalescent solution became water-clear. After about four hours' dialysis against 0.012 M tris buffer pH 8.5, which resulted in the lowering of pH to slightly below 10, the protein solution was centrifuged at 150 000 g for 60 min to remove intact virions. RNA was removed by passage through a small column ($\pm$ 30 ml bed volume) of Whatman DE52 DEAE-cellulose which had been pre-equilibrated against 0.12 M tris buffer pH 8.5. This buffer was also used to elute the protein from the column. After discarding a volume of fluid equivalent to the void volume, 20 ml samples of effluent were collected and monitored for TMVP by means of optical density (OD) measurements at 282 nm. Samples containing significant amounts of protein, and having the desired OD_{282}: OD_{251} ratio (Durham, 1972a; also Results, section A2) were pooled and dialysed against 0.05 M acetate buffer pH 5.0 for at least 24 h. At pH 5.0 the preparation became opalescent due to the formation of protein helices. These could be concentrated by centrifugation at 150 000 g for 60 min and the pellet re-suspended in 0.12 M tris buffer pH 8.5. At this stage, protein obtained from the different strains of TMV had characteristic UV spectra (see Results, section A2) which were indicative of virtually complete removal of RNA by the ion exchange column. TMVP was always stored at $4^{o}C$ in the helical form in buffer at pH 5.0 at which hydrogen ion concentration it has been found to be the most stable (Durham, 1972a).

TMVP was occasionally prepared from the four strains by the acetic acid method of Fraenkel-Conrat (1957). The UV absorbance characteristics of these proteins were indistinguishable from those of proteins prepared by the ethanolamine method.

4. Virus and protein quantitation

Virus and protein preparations were routinely quantitated by means of ultraviolet spectroscopy. A prerequisite was an accurate extinction coefficient for each TMV strain and TMV protein. Extinction coefficients were thus determined, and those reported in the literature confirmed, as follows: After exhaustive dialysis of purified virus or protein preparations against dilute electrolyte (usually 1 mM or 10 mM KCl), aliquots of 3.0 or 4.0 ml of the dialysed preparation (and of the dialysate as blank) were dried over silica gel in a heated vacuum dessicator operating at 95^{o}C, until constant weight was attained. Simple subtraction of the blank figure yielded the solute concentration. Alternatively, the refractive indices of the preparations and of the dialysates were determined to six decimal places. This was done with a Carl Zeiss immersion refractometer, with a waterbath controlling the temperature to within 0.1^{o}C. Thus the refractive index contribution of the macromolecular solute alone could be obtained and converted to a concentration figure using a specific refractive index increment coefficient for TMV of 0.1852 x 10^{-3} $(mg/ml)^{-1}$ (Scheele & Lauffer, 1967). Having determined the concentration, the optical density of a solution at a suitable dilution in phosphate buffer pH 7.0 of ionic strength I=0.05 was determined at either 260 nm or 282 nm. Readings were then made at each 20 nm interval from 340 nm to 500 nm, using a Unicam SP700 spectrophotometer. These readings were corrected for instrument and cuvette artifacts by subtracting blank readings obtained over the same wavelength range. The OD_{260} or OD_{282} reading was corrected for light scattering by extrapolation into this region of the regression line obtained from a plot of log OD versus log of wavelength over the range 340 nm to 500 nm (Bonhoeffer & Schachman, 1960). The corrected OD value was used in conjunction with the predetermined concentration to calculate the extinction coefficient (see Results, section A3, and also Appendix 1 for an example of an OD determination).

The same spectrophotometric procedure outlined above was used to determine the OD whenever a fresh TMV preparation had to be quantitated. Using the extinction coefficient, the OD was converted to virus or protein concentration in mg/ml, and then to molar concentration of TMVP, assuming a molecular weight of 17 500 for TMVP (Hennig & Wittmann, 1972) and an RNA content of 5.0% for TMV (Knight & Woody, 1958). All quantitative results could then be expressed on a "per TMVP subunit" basis.

Preparations of BMV were quantitated, as for TMV, assuming a corrected extinction coefficient ($E^{1mg/ml}_{260\ nm}$) of 5.08 (Bancroft, 1970a), an RNA content of 22% (Bockstahler & Kaesberg, 1962) and a protein subunit molecular weight of 20 300 (Stubbs & Kaesberg, 1964). TCV, with an RNA content of 17% (Symons _et al._, 1963) and a protein subunit molecular weight of 41 000 (Ziegler _et al._, 1974), was found to have an $E^{1mg/ml}_{260\ nm}$ of 5.0 (A.C.H. Durham, unpublished results). TYMV contains 34% RNA (Kaper & Litjens, 1966), and has a protein subunit molecular weight of 20 100 (Symons _et al._, 1963; Harris & Hindley, 1965) and an $E^{1mg/ml}_{260\ nm}$ of 8.6 (Kaper & Litjens, 1966). The above data were used to convert the OD_{260} of solutions of these viruses to concentration figures expressed in terms of molarity of the protein subunits.

B. Virus dissociation induced by cation removal

Divalent cations were removed from TMV particles by chelation with EDTA in an attempt to induce _in vitro_ virus dissociation. This was done to test the possibility that divalent cations were essential for virus stability and that they acted as a trigger for dissociation. Virus was either suspended in or dialysed against EDTA solutions, of which four parameters were varied:

(a) the EDTA concentration, which ranged from 1 to 50 mM;

(b) the calcium concentration, which was regulated at levels from 10^{-3} M (pCa 3) to 10^{-9} M (pCa 9) by using the EDTA as a pCa buffer (Perrin & Dempsey, 1974);

(c) the ionic strength (I), which was adjusted to values ranging from I=0.004 to I=0.1 by the addition of KCl, as the degree of ionisation and therefore the ionic strength of a given EDTA solution varies with pH (see Table 2); and

(d) the pH, which was adjusted to values between pH 7.0 and pH 8.5 by the addition of NaOH to the EDTA solutions. However, above pH 8 and particularly in the presence of Ca^{2+}, EDTA has minimal pH buffering capacity. When EDTA is used as a pCa buffer, a second non-interfering buffer can be added to control the pH (Perrin & Dempsey, 1974).

TABLE 2

The ionic strength of EDTA solutions as a function of pH[a]

pH	Ionic strength (I)							
	EDTA concentration (mM)							
	1	2	5	10	15	30	50	100
7.0	0.00419	0.00838	0.0209	0.0419	0.0627	0.1254	0.209	0.419
7.5	0.00439	0.00878	0.0219	0.0439	0.0657	0.1314	0.219	0.439
8.0	0.00448	0.00896	0.0224	0.0448	0.0672	0.1344	0.224	0.448
8.5	0.00454	0.00908	0.0227	0.0454	0.0681	0.1362	0.227	0.454
9.0	0.00467	0.00934	0.0233	0.0467	0.0699	0.1398	0.233	0.467

[a] Durham, 1975.

Purified type strain of TMV, at 5 to 10 mg/ml in 0.02 M phosphate buffer of pH 7.4, was initially freed of accompanying dissociated protein. This was done either by centrifuging virus at 27 000 r.p.m. for 2 h in a gradient of sucrose ranging from 5 to 30% using a Beckman SW 27 rotor and then isolating the virus which showed up as an opalescent band, or by pelleting the virus by centrifugation at 150 000 g for 60 min. The virus sample obtained from the gradient experiment was diluted, or the virus pellet re-suspended in the appropriate EDTA buffer against which the virus was subsequently dialysed at temperatures ranging between 4^{o} and $22^{o}C$. The time of dialysis varied from 0.5 to 120 h. To assay the extent of protein dissociation induced by this dialysis, the intact virus particles were removed from solution by centrifugation at 135 000 g for 180 min. A SW 27.1 swing-out rotor was used to minimise virus adhesion to the walls of the tubes. The rotor was allowed to coast to a halt to minimise re-suspension of the pellet, and the top half of the supernatant was carefully removed using a Pasteur pipette.

The supernatants were assayed for dissociated protein by a ninhydrin colorimetric method (Chase & Williams, 1968). This method was preferred as both EDTA and Ca^{2+} ions interfered with the Lowry (Lowry et al., 1951) method of protein assay. Duplicate aliquots of 0.5 ml of each supernatant, as well as of appropriate EDTA and distilled water blanks and of standard TMV solutions ranging from 5 to 150 μg/ml, were placed in polyethylene tubes, 1 ml N NaOH added to each, and the tubes were heated in a $100^{o}C$ oven for 5 h. After cooling for 5 min, 1 ml 30% acetic acid was added, and the tubes submerged in boiling water for 6 min. After adding 1 ml ninhydrin-hydrindantoin reagent to each tube and checking that the pH was 5, the tubes were again subjected to the heat treatment for 15 min. Five ml 50% ethanol was added to each, and the tubes were cooled by dunking the rack in cold water. The contents were mixed by inversion and, after standing at room temperature for 15 min, their absorbances at 570 nm were read versus distilled water.

C. Equilibrium dialysis

The binding of calcium to TMV as a function of pH and pCa was determined directly by equilibrium dialysis. For this purpose, a rotatable multichamber apparatus, capable of accommodating 1 ml samples, was drilled out of polymethylmethacrylate. Virus or protein solutions (freed of divalent cations as described in section E1 below), at concentrations of 5 to 10 mg/ml, were dialysed against two changes of a buffer of chosen pH and pCa. Depending on the pH required, 10 mM acetate, 2-(N-morpholino) ethane sulphonate, histidine, phosphate, imidazole or trishydroxymethylaminomethane buffers were used. Appropriate free Ca^{2+} ion concentrations were obtained by addition of volumetric 1 M $CaCl_2$ (BDH, Poole, England) and were buffered with nitrilotriacetic acid. The ionic strength was adjusted to I=0.1 with KCl.

Samples of 1 ml of virus or protein were placed on one side of the dialysis membrane, and dialysate on the other. A small volume of $^{45}CaCl_2$ solution (Radiochemical Centre, Amersham, England) was added and the apparatus was rotated at $6^oC \pm 2^oC$ for 48 h. A precision microlitre syringe (Hamilton Instruments, Whittier, California) was used to remove 200 μl samples from each side. These samples were then added to 10 ml of a scintillation cocktail (Monophase 40, from Packard Instruments, Zurich, Switzerland) in a polypropylene vial (Beckman Instruments, Palo Alto, California) and radioactive disintegrations were measured in a Packard Model 3385 scintillation counter using the ^{14}C preset energy window.

The attainment of dialysis and chemical equilibrium was proved by the agreement between duplicate experiments in which the ^{45}Ca was added to opposite sides of the dialysis membrane, and by their agreement with trial experiments lasting more than a week. Quenching, adsorption to the vials, and precipitation of salts were found not to be significant sources of radioactive counting errors. Virus and protein concentrations were determined spectrophotometrically at the end of the experiment.

The number of calcium ions bound per subunit was calculated using the equation:

$$\frac{[Ca^{2+}]\ \text{bound}}{[Ca^{2+}]\ \text{free}} = \frac{cpm_{virus} - cpm_{dialysate}}{cpm_{dialysate} - cpm_{background}}$$

$$Ca^{2+}\ \text{ions/subunit} = \frac{cpm_{virus} - cpm_{dialysate}}{cpm_{dialysate} - cpm_{background}} \cdot \frac{[Ca^{2+}]\ \text{free}}{[\text{subunit}]}$$

However, a correction factor for the volume occupied by the virus or protein had to be included in this equation, which then became:

$$Ca^{2+}\ \text{ions/subunit} = \frac{cpm_{virus} - cpm_{dialysate} + Q}{cpm_{dialysate} - cpm_{background}} \cdot \frac{[Ca^{2+}]\text{free}}{[\text{subunit}]}$$

where Q = virus or protein concentration (in g/ml) x $\bar{V}$ x ($cpm_{dialysate}$ - $cpm_{background}$).

Partial specific volumes ($\bar{V}$) of 0.71 and 0.73 were assumed for TMV and TMVP respectively (Lauffer & Stevens, 1968; Durham, 1972a).

D. Determination of bound calcium by sedimentation

Bound calcium was also determined using a sedimentation method similar to that of Cohen & Selinger (1969). This technique has been discussed by Cann (1970) and by Van Holde (1975). As stressed by Kretsinger & Nelson (1976), it can only be employed if the complex is readily sedimented, as in the case of membrane fragments or virus particles.

Solutions of the four virus strains from which bound cations had been removed (see section E1 below), at virus concentrations of between 8 and 10 mg/ml, were dialysed for 48 h at 22°C against either pH 6.0 cacodylate, pH 7.0 imidazole or pH 8.0 tris buffers. These had final ionic strengths of I=0.16 obtained by the addition of KCl to I=0.01 buffer solutions. After dialysis, the virus concentrations were determined spectrophotometrically. For each TMV strain at a particular pH, a range of 5 dilutions, each of 900 μl final volume, was made using small plastic vials. A sixth vial contained 900 μl of dialysate. To each vial was added 100 μl of 10^{-2} M $CaCl_2$ solution, containing about 0.3 μCi of $^{45}CaCl_2$ per ml and adjusted to I=0.16 with KCl. The concentrations of the reactants were adjusted to obtain final virus concentrations ranging between 0.8 and 5.5 mg/ml, a final calcium concentration of 10^{-3} M, and sufficient radioactivity in the final solutions to yield counts of at least 30 000 cpm using samples of 250 μl. Alternatively, 200 μl of $CaCl_2$ solution was added to 800 μl of virus solution if calcium concentrations of 2×10^{-3} M were desired. In a control experiment performed without radioactive calcium, the addition of 200 μl of a 10^{-2} M $CaCl_2$ solution to 800 μl of pH 7.0 buffered virus solution at a concentration of 4.5 mg/ml resulted in no detectable alteration in the pH.

After 12 h at 22°C with occasional agitation, 680 μl volumes were removed from each of the six vials using an adjustable microlitrepipette (Finnpipette, Finland) and centrifuged at 40 000 rpm for 2 h in a 6-bucket Beckman SW 50 rotor fitted with small volume tubes and adaptors. After centrifugation, 250 μl was transferred from the supernatant of each tube to a polypropylene counting vial, 750 μl of distilled water and 10 ml of scintillation cocktail was added to each, and radioactive disintegrations were measured as described for the equilibrium dialysis experiments. Spectrophotometric examination of a supernatant obtained after centrifugation of a solution that had contained 4.5 mg virus/ml yielded OD_{260} and OD_{280} readings of 0.008 and 0.004 respectively, indicating that the centrifugation step was resulting in virtually complete removal of the virus from the supernatant.

The counts obtained with the centrifuged dialysate blanks were regarded as representing the total original calcium concentrations. The supernatants from the tubes that had contained virus always yielded lower counts than the blank, due to bound ^{45}Ca having been removed with the sedimented virus, the difference between the two counts being proportional to the amount of calcium bound. This difference in calcium concentration (in μM units) was plotted against the μm concentration of virus protein subunits (see section A4 above), and regressional analyses of the linear plots yielded slopes equivalent to Ca^{2+} ions bound per protein subunit. A specimen calculation is presented in Appendix 2.

E. Titration

The binding of divalent cations to virus or protein was monitored potentiometrically by titrating the protons displaced when a given amount of a divalent cation was added to a known amount of virus or protein. This was done either by titrating the protons displaced by a known amount of cation from an aliquot of material at a specific pH, or by obtaining the differential curve between titration in the absence and in the presence of a specific divalent cation respectively, obtained over a given pH range. An obvious prerequisite to this type of examination was the removal of all adventitiously bound divalent cations from the material under study.

1. Removal of bound cations

Bound divalent cations, particularly Ca^{2+} and Mg^{2+}, were removed from purified virus or protein by dialysis against EDTA. Volumes of up to 100 ml of material at concentrations between 5 and 10 mg/ml were dialysed overnight at $4^{o}C$ against 2 l of 50 mM EDTA adjusted to pH 7.5 with NaOH. This pH was chosen despite the greater stability of certain viruses such as BMV at a lower pH. The reason for this is the greater affinity of EDTA for divalent cations at higher pH. It is recognised (see e.g. Perrin & Dempsey, 1974) that EDTA has a 100-fold greater affinity for Ca^{2+} at pH 8.0 than it has at pH 6.0.

As traces of EDTA would interfere with potentiometric titrations, care had to be taken that EDTA was removed quantitatively by exhaustive dialysis. For this purpose, a volume of 50 mM KCl was used as dialysate instead of distilled water, in order to provide salt counter-ions for the virus or protein. This concentration of KCl was chosen because in 100 mM KCl solution TMV precipitated at pH 4.5. Merck analytical grade KCl was dissolved in deionised water that had been redistilled over quartz. The pH was adjusted to approximately 5 with a few drops of HCl to minimise solubilization of atmospheric CO_2. All dialyses were performed at 4°C in polyethylene vessels using magnetic stirrers. The KCl solution was changed every 24 h and a minimum of 5 changes of 2 to 4 l each was used. Upon completion of dialysis, preparations were stored at 4°C in thoroughly washed polyethylene bottles.

2. Confirmation of divalent cation removal

Samples of all the purified strains, both before and after undergoing the EDTA/KCl dialysis treatment described above, as well as samples dialysed against 50 mM KCl only, were examined for the presence of bound Ca^{2+} and Mg^{2+} ions by atomic absorption spectroscopy. These analyses, performed on a Varian Techtron AA-6 atomic absorption spectrometer by Mr J. Marchant and Mr B. Kloppers, Geochemistry Department, University of Cape Town, confirmed that EDTA dialysis was removing bound cations quantitatively (see Results, section B3).

3. Confirmation of EDTA removal

The absence of residual EDTA was confirmed by centrifuging dialysed virus samples, adjusted to pH 7.5 with NaOH, in a SW 50.1 swing-out rotor at 300 000 g for 50 min. The tubes contained a 20% sucrose cushion to prevent redistribution of the sedimented virus particles. The supernatants were examined by potentiometric titration for residual EDTA. It was found routinely after the dialysis against KCl that no EDTA was detectable in the supernatants, either by the presence of a typical EDTA titration curve (see section E8 below) or by a measurable proton release (i.e. lowering of pH) after the addition of Ca^{++}.

4. Virus degradation during dialysis

The supernatants (see previous section) were also examined spectrophotometrically for the presence of dissociated virus protein. UV absorbance readings indicated that, in the case of the type, U2 and cowpea strains of TMV, usually less than 0.3% of the total material in the centrifuge tube was present as dissociated protein, indicating that insignificant dissociation of these strains had occurred during the dialysis procedure. This figure was consistently higher in the case of the Y-TAMV strain, probably due to the virions being less stable then those of the other strains. In immunodiffusion tests with homologous antiserum, Y-TAMV regularly produces, in addition to the virus band, a precipitin band due to dissociated protein (van Regenmortel, unpublished results). Confirmation of the presence of dissociated protein in preparations of Y-TAMV that had been dialysed against EDTA was obtained by centrifugation of Y-TAMV at 135 000 g for 2 h on 0% to 30% sucrose density gradients in 50 mM phosphate buffer plus 2 mM EDTA at both pH 6.5 and pH 7.5. The gradients were fractionated by the removal of 2 ml samples from the top of the tube and the samples were monitored at 282 nm for the presence of UV light absorbing material. The tubes containing Y-TAMV had UV absorbing material present at the meniscus after centrifugation, while those containing type strain as control showed no evidence of non-sedimenting, UV absorbing material (Fig. 1).

After the dialysis procedure, Y-TAMV was thus pelleted by centrifugation at 150 000 g for 60 min, resuspended in dialysate and used immediately afterwards. Similar protein dissociation was detected after dialysis of the spherical

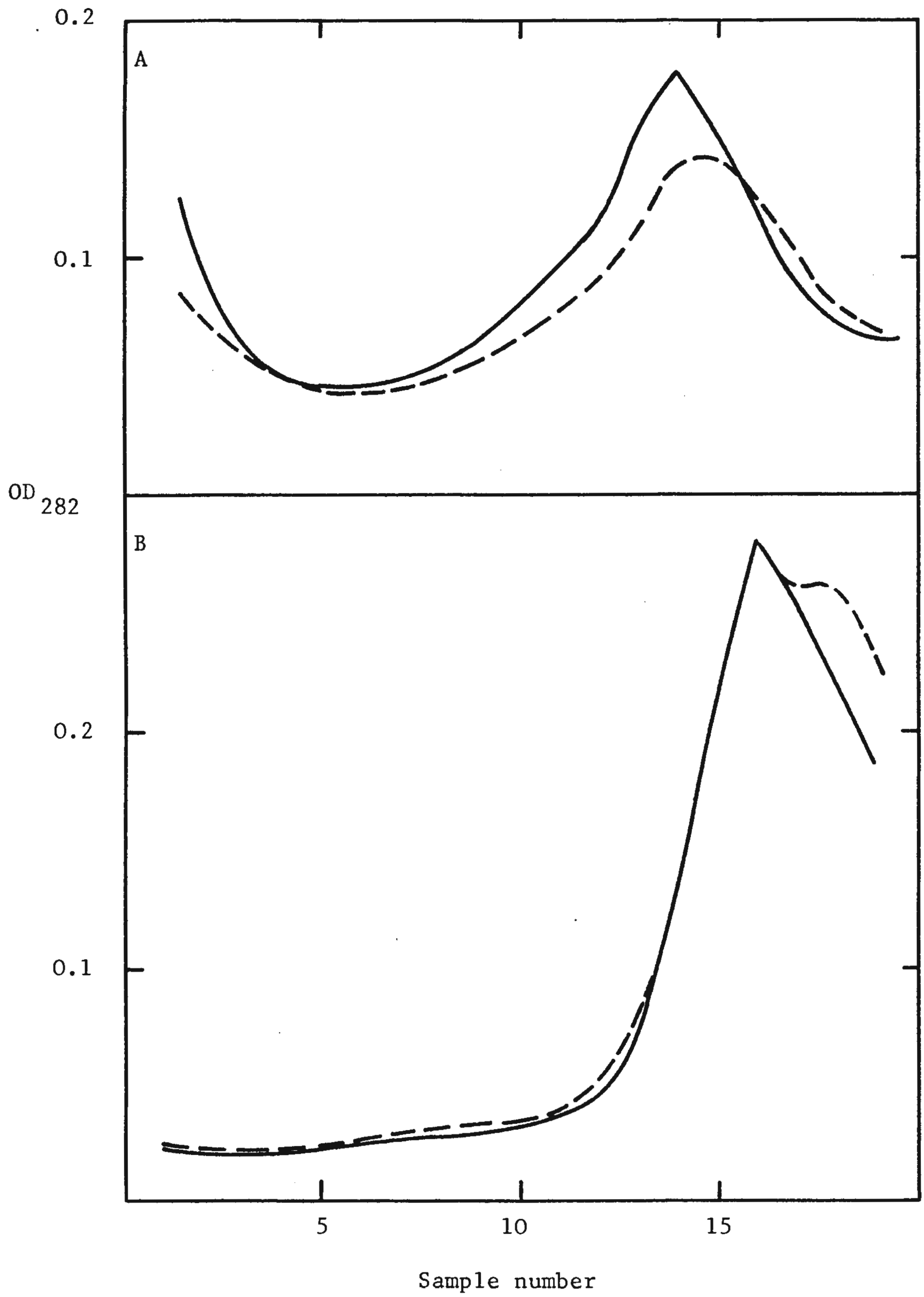


Figure 1. Scanning patterns of centrifuged density gradient columns. Samples of 1.2 mg of Y-TAMV (A) and of 2.0 mg of type strain (B) were centrifuged at pH 6.5 (solid line) and at pH 7.5 (dotted line). Volumes of 2 ml were sampled from the top of the tube, diluted with 1 ml distilled water and the O.D. determined at 282 nm. The meniscus is on the left in both cases. Note the presence of UV absorbing material near the meniscus of A.

viruses BMV and TCV. After dialysis, preparations were quantitated as in section A4 above.

5. Preparation of titrants and cation solutions

The titrants used were 10 mM NaOH and 10 mM HCl diluted from standard volumetric solutions (Merck, W. Germany), each then being adjusted to contain 50 mM KCl. Occasionally, 40 mM titrants were used for more concentrated virus or protein solutions. Cations were added, to solutions being titrated, as 1 M or 100 mM solutions of their chloride salts. In addition, these cation solutions contained 50 mM KCl. $CaCl_2$ was diluted from standard volumetric solutions (AVS Volumetric solution, BDH, Poole, England), while $MgCl_2$, $BaCl_2$, $SrCl_2$, $MnCl_2$ and $LaCl_3$ solutions were made by weighing out hydrated, analytical grade solids. Lead was added as Pb $(NO_3)_2$, with the omission of KCl as $PbCl_2$ is only barely soluble in water.

6. Apparatus and methods of titration

All titrations were performed using Radiometer equipment (Copenhagen, Denmark) installed in an air-conditioned room which was maintained at $22^{\circ} \pm 1^{\circ}$C. This included a PHM 64 three decimal place digital pH meter, a TTT 60 autotitrator, a TTA 31 titration assembly with magnetic stirrer and semi-micro polythene vessels, an ABU 11 and an ABU 12 2.5 ml auto-burette, and a GK2320 C combined glass-calomel electrode.

Carbon dioxide was excluded from the titration vessel by blowing over the liquid surface a stream of air that had been passed slowly through a column of soda lime granules followed by bubbling through a concentrated sodium hydroxide solution. The pH meter was standardised daily using phosphate (pH 6.87) and phthalate (pH 4.00) standard buffers. Whenever the electrode began showing a lag in response time, which could have been the result of denatured protein adhering to the porous plug, it was rejuvenated by alternate rinses with

0.1 N HCl and 0.1 N NaOH. Separate glass (G2222c) and calomel (K4112) electrodes were used, the latter in conjunction with a saturated KNO_3 solution second-salt-bridge, for titration in the presence of lead ions. This electrode arrangement required careful earthing of all the components in order to obtain stable pH readings.

Prior to titration, and also after dilution to the desired concentration, virus and protein preparations were allowed to equilibrate slowly to room temperature. The polymerisation of TMVP is enhanced by increasing protein concentration (Banerjee & Lauffer, 1966). Samples of 5.0 ml of virus or protein at concentrations of 3 to 5 mg/ml, equivalent to 170-290 µM TMVP, were used for each titration. At these concentrations, a titration in one direction between pH 4.5 and pH 8.5 consumed between 500 and 1000 µl of 10 mM titrant. By using a combined electrode and operating at room temperature, this arrangement had the advantage over those of e.g. Butler et al. (1972) and Paulsen (1972) that salt solutions could be added to the titration vessel using Eppendorff (Hamburg, Germany) pipettes and that the virus solution could be observed during titration. Titrations were usually commenced with the solution at pH 4.50 and successive 10 µl amounts, or multiples thereof, of alkaline titrant were added using the automatic increment facility of the ABU 11 and ABU 12 burettes. After each addition, the pH was recorded once the reading had not fluctuated by more than 0.002 pH units for at least one min. This equilibrium usually took a few minutes, but could take up to one hour in certain cases, e.g. with TMVP in the region of pH 7. Upon completion, usually near pH 8.5, a back titration to pH 4.5 was performed with acid titrant to test for reversibility and hysteresis.

For titrations in the presence of a specific cation, either 100 µl of a 100 mM solution or 200 µl of a 1 M solution were added to the sample at a specific pH. These amounts resulted in final cation concentrations in the vessel of about 1.5 mM and 27 mM, corresponding to about 10 and 200 cations per protein subunit, respectively. The first level was arbitrarily chosen, while the second was chosen because it was found that in the case of TMV vulgare the addition of more calcium solution resulted in negligible further proton displacement. The cation added caused a rise in ionic strength from I=0.05 to, at most, I=0.17. After the addition of cation, the amount of titrant required to restore the original pH was noted for subsequent alignment of the differential plot (see following section), and the pH was adjusted to the desired starting value (usually pH 4.5) for the titration. The above procedure was followed whenever the proton displacement by a cation at a definite pH was determined, even if a subsequent titration was not performed. These proton displacement values at specific pH's were essential for the correct positioning of titration curves obtained in the presence of cations, relative to the basic curve obtained without cations. All titrations were repeated on a minimum of 3 aliquots to test for reproducibility.

Similar titrations from pH 4.5 to pH 8.5 were performed on 5 ml aliquots of dialysate, both with and without added cations, to obtain blank correction figures. This correction was particularly important in the case of lead and manganese, which tend to hydrolyse (i.e. the hydrated ions act as proton donors) at and above neutrality (Gurd & Wilcox, 1956).

7. Plotting and standardising of data

An example of the plotting and standardising of titration data is presented in Appendix 3. From a plot of titrant volume (ordinate) versus pH (abscissa), adjusted vertically to pass through the origin at pH 4.500, the volume of titrant added was noted for every 0.2 pH interval. After these volumes had been corrected for minor deviations in the titrant from the desired 10 mM or 40 mM concentration (see section 8(d) below), appropriate blank figures (obtained from a titration of dialysate alone) were subtracted and the corrected volumes thus obtained were converted stoichiometrically to protons titrating per subunit. This conversion was based on the known virus or protein concentration and the concentration of the titrant (i.e. 10 or 40 mM). When forward and reverse titration plots had to be superimposed, e.g. to illustrate hysteresis, particular attention was paid to correcting for minor deviations in the concentration of either titrant.

Titrations were used to quantitate hysteresis by obtaining differential plots between forward and reverse titration curves. The difference in titratable protons between the reverse and forward curves at every 0.2 pH interval was obtained by simple subtraction, and plotted as a function of pH.

Titrations were also used to quantitate the protons displaced by a cation from virus or protein. At each 0.2 pH interval, the difference in titratable protons between the curve obtained in the presence of cation and that obtained in the absence was derived by simple subtraction. This difference represented protons displaced as a result of cation binding to the virus or protein, and was plotted as a function of pH. This differential plot was accurately aligned vertically using actual determinations of protons displaced at a specific pH by the cation; these were derived from the amounts of titrant required to restore the pH of an aliquot of material to which cation had been added (see section B6 above).

8. Precautions

Various precautions (some already mentioned) had to be observed to minimise titration errors. These were:

(a) CO_2 exclusion. Dissolved carbon dioxide goes into solution as H_2CO_3, with pK_1 of 3.8 and pK_2 of 6.4 (Sober, 1970). Titrations were thus performed under CO_2-free air, and dialyses were performed at pH 5, where possible (see section E1 and E6 above).

(b) Bubbles in the titrant lines. The formation of gas bubbles in the titrant lines, due to dissolved gases emerging from solution, was a major problem. These were dislodged either by tapping the vertically held line, or by "sweeping" the line by pumping through a large bubble introduced from the burette. Expansion of these bubbles resulted in slow expulsion of titrant at the nozzle which caused a slow drift in pH.

(c) Efficient stirring. This was a problem particularly below pH 5, where certain virus strains or their proteins tended to form viscous "clumps" upon addition of acid titrant. These clumps, which were due to localised regions of isoelectric precipitation, occasionally lodged unnoticed in inadequately stirred regions of the vessel. Their slow resolution resulted in pH drift. This was prevented by increasing the stirring rate, or by arranging the electrode and nozzles so as to prevent "corners" where the clumps might be trapped.

(d) Relative titrant strengths. Poor correspondence of forward and reverse titration curves was obtained if corrections were not introduced for inequalities in acid and alkali titrant strengths. Potentiometric titration of the acid against the alkali yielded a correction factor.

(e) EDTA removal. Residual EDTA was detected in dialysates or supernatants (see section E3 above) by the production of a titration curve with points of inflection near pH 6.2 and pH 10.3, being the pK_1 and pK_2 respectively of EDTA (Perrin & Dempsey, 1974). A pH lowering upon addition of Ca^{2+} also indicated the presence of EDTA.

(f) Virus degradation. This occurred either during dialysis or during titration. The former (see section E4 above) resulted in the generation of dissociated protein subunits which in turn caused TMV titration curves to show spurious hysteresis loops (see Results, section C1). Degradation during titration, which generally occurred at elevated pH's or after protracted titrations, possibly accounted for the poor correspondence occasionally observed between forward and reverse titrations.

(g) Electrostatic effects. These were minimal with the combined electrode, but had to be compensated for when separate glass and calomel electrodes were used (see section E6 above).

(h) pH drift. This could generally be ascribed to either inadequate stirring, bubble formation in the titrant lines, CO_2 leakage, protein denaturation, or temperature drift of the sample due to inadequate equilibration to room temperature.

(i) pH meter lag. Slow electrode response, presumably due to protein denaturation on the porous plug, was corrected by alternate acid-alkali rinsing of the electrode. When not in use, the electrode was kept immersed in phthalate buffer of pH 4.

(j) pH meter standardisation. Standardisation was done daily using standard phosphate and phthalate buffers maintained at $4^{\circ}C$. Aliquots were equilibrated to room temperature before use. The daily adjustment seldom exceeded 0.01 pH units.

F. Analytical ultracentrifugation

A Beckman Model E analytical ultracentrifuge equipped with an electronic speed control was used to examine the effect of the presence of Ca^{2+} on the pH-induced aggregation states of TMV protein (Durham, 1972a). Protein was prepared from the type, U2, Y-TAMV and cowpea strains of TMV as described in section A3 above. Adventitiously-bound divalent cations were removed as described in section E1 above. Aliquots of protein, adjusted to 5 mg/ml in 50 mM KCl, were dialysed in the cold for at least 48 h against imidazole buffers of the desired pH and Ca^{2+} concentration. The ionic strength of the buffers was kept constant at I=0.17 by the addition of KCl. A dialysis period of 48 h was found by Durham (1972a) to be adequate for the attainment of equilibrium.

Upon completion of dialysis, the pH of the buffer was checked and the protein was equilibrated at room temperature (21^{o}C) for at least 12 h. Two samples were examined per run using standard and 1^{o} positive wedge windows with an An-D rotor operating at speeds from 18 000 to 56 000 r.p.m. at temperatures between 20 and 22^{o}C. Photographs were taken on Kodalith Ortho film type 3 using schlieren optics, and the positions of the sedimenting peaks were measured using a Nikon 6CT2 microcomparator. The sedimentation coefficients calculated were not corrected for temperature and buffer viscosity, the small corrections being insignificant in comparison to the large differences between the sedimentation coefficients of the various protein aggregates which were of interest in this study.

Imidazole buffers were chosen for the dialysis, being best suited to the pH range of interest, viz. pH 6.2 to pH 7.5. Although imidazole can act as a metal ion ligand under certain conditions (Vallee & Wacker, 1970), it has a negligible affinity for Ca^{2+} ions at I=0,16 and neutral pH (Schubert, 1954).

CHAPTER FOUR

RESULTS

A. Identification and quantitation of material

1. Identification of TMV strains

A confirmation by amino acid analysis of the identities of the four TMV strains used in the present study was an essential prerequisite to any experimentation. However, as the object was identification of the strains and not the determination of their complete amino acid compositions, an accurate determination of the amounts of all amino acids was not necessary. The amounts of serine and threonine residues obtained in the analysis were not included in the calculation, as these amino acids are degraded to varying extents during acid hydrolysis (Knight, 1975). The calculated amounts of lysine, histidine, aspartic acid, glutamic acid, alanine, methionine and leucine (Table 2) confirmed the identities of the four TMV strains.

2. Protein characterisation

The spectral characteristics determined for the proteins of the type, U2, Y-TAMV and cowpea strains of TMV are listed in Table 3. Typical UV absorbance spectra of each protein are illustrated in Figure 2.

3. Virus and protein extinction coefficients

Virus extinction coefficients based on concentrations determined by dry weight measurements are presented in Table 4, while those based on concentrations determined refractometrically are listed in Table 5. Protein extinction coefficients obtained using solutions that had been quantitated refractometrically are presented in Table 6.

TABLE 3

Amino acid analysis of selected residues of the proteins of four TMV strains

Amino acid	Vulgare Residues determined	Vulgare Actual number[a]	Y-TAMV Residues determined	Y-TAMV Actual number[b]	U2 Residues determined	U2 Actual number[a]	Cowpea Residues determined	Cowpea Actual number[c]
trp	2.44	3	2.30	3	1.55	2	1.06	1
lys	2.17	2	2.14	2	1.07	1	1.31	1
his	0	0	0	0	0	0	1.00	1
arg	12.41	11	10.35	9	9.06	8	13.49	12
asp	17.79	18	17.91	18	21.90	22	18.08	18
glu	16.20	16	18.51	19	16.15	16	16.34	16
pro	10.45	8	10.32	8	12.40	10	8.37	8
gly	5.92	6	5.75	6	-	-	4.02	4
ala	13.94	14	11.45	11	16.84	17	11.02	12
val	12.91	14	13.91	15	11.22	12	11.12	12
met	0	0	0.85	1	1.96	2	0.34	0
ile	7.36	9	5.83	7	6.46	8	9.04	10
leu	11.90	12	12.57	13	10.95	11	15.19	15
tyr	3.80	4	4.53	5	5.71	6	7.80	8
phe	7.68	8	7.58	8	7.67	8	5.84	6
Total	124.97	125	124.00	124	122.94	123	124.02	124

[a] Fraenkel-Conrat (1974)

[b] Knight *et al.* (1962)

[c] Rees & Short (1965)

TABLE 4

Spectral characteristics and aromatic amino acid compositions[a] of the proteins of four TMV strains

	Type	Y-TAMV	U2	Cowpea
λ_{max} (nm)	282	282	281	279
λ_{min} (nm)	251	251	251	251
$\frac{OD_{max}}{OD_{min}}$	≥ 2.5	≥ 2.8	≥ 3.2	≥ 4.0
Σtrp	3	3	2	1
Σtyr	4	5	6	8
Σphe	8	8	8	6

[a]See Table 3.

4. Discussion

The amino acid analyses were performed on samples that had been hydrolysed for 24 h at 110°C. Longer hydrolyses were not attempted, although these might have improved the low yields obtained with isoleucine and valine. The former amino acid forms very stable bonds, particularly with contiguous isoleucine and valine residues (Knight, 1975). Threonine and serine residues were degraded by the acid hydrolysis (Knight, 1975), and their amounts were thus not included in Table 3. The reason for the high numbers of arginine and proline residues is not known, although it is recognised that aberrant results with particularly the former amino acid are common (Eastoe, 1966; Leggett Bailey, 1962).

TABLE 5

Virus extinction coefficients based on dry weight concentration determinations

TMV strain	Dry weight determinations			Optical density characteristics[f]							$E_{260\ nm}^{1\ mg/ml}$ [e]
	Starting volume (ml)	Virus dry weight (mg)[a]	Virus conc. (mg/ml)	$\frac{OD_{max}}{OD_{min}}$ [b]	$\frac{OD_{280\ nm}}{OD_{260\ nm}}$	Light scattering plot[c] Slope	Light scattering plot[c] Correl. coeff.	Observed $OD_{260\ nm}$	Corrected $OD_{260\ nm}$ [d]	Dilution factor	
U2	3	20.80±0.07	6.94±0.02	1.19	0.84	-2.83	0.994	0.388	0.340	50	2.45
Cowpea	4	27.65±0.11	6.91±0.03	1.27	0.88	-4.14	0.998	0.360	0.298	50	2.16
Y-TAMV	3	32.09±0.10	10.68±0.03	1.25	0.87	-4.05	0.998	0.323	0.266	100	2.49

[a] For each strain, duplicate samples of dialysed material were dried for at least 4 days, with daily weighing, and the constant weights thus obtained were corrected for the electrolyte content of the dialysate (0.30 g KCl/4 l = 0.075 mg/ml). Standard deviations are given.

[b] The OD maxima and minima, which were at 260 nm and 248 nm resp. for all 3 strains, were corrected for light scattering.

[c] A Compucorp Statistician desk calculator was used to obtain the slope and correlation coefficient of the regression line of a plot of log OD vs. log wavelength over the range 340 nm to 500 nm.

[d] The light scattering at 260 nm was obtained by computed extrapolation of the scattering regression line, and subtracted from the observed $OD_{260\ nm}$.

[e] $$E_{260\ nm}^{1\ mg/ml} = \frac{\text{corrected } OD_{260\ nm} \times \text{dilution factor}}{\text{concentration}}$$

[f] OD data were obtained using suitable dilutions, in I=0.05 phosphate buffer pH 7.0, of the dialysed material used for the dry weight determinations.

TABLE 6

Virus extinction coefficients based on refractometric concentration determinations

TMV strain	Refractive index determinations: Temp.	$n_{solvent}$ [a]	$n_{solution}$ [a]	Δn	Virus conc.[g] (mg/ml)	Optical density characteristics[f]: $\frac{OD_{max}}{OD_{min}}$ [b]	$\frac{OD_{280\ nm}}{OD_{260\ nm}}$	Light scattering plot[c]: Slope	Correl. coeff.	Observed $OD_{260\ nm}$	Corrected[d] $OD_{260\ nm}$	Dilution factor	$E^{1\ mg/ml}_{260\ nm}$ [e]
Vulgare	22.0 21.2	1.33293 1.33301	1.33486 1.33490	0.00193 0.00189	10.31±0.20[h]	1.25	0.86	-3.75	0.999	0.633	0.532	50	2.58
U2	22.0 21.2	1.33293 1.33301	1.33645 1.33655	0.00352 0.00354	19.06±0.14	1.18	0.84	-3.61	0.998	0.543	0.457	100	2.40
Cowpea	21.2 22.0	1.33301 1.33293	1.33643 1.33815	0.00344 0.00342	18.52±0.14	1.25	0.87	-3.74	0.999	0.497	0.412	100	2.22
Y-TAMV	22.0 21.2	1.33293 1.33301	1.33815 1.33823	0.00522 0.00522	28.19±0.08	1.24	0.86	-3.80	0.998	0.425	0.352	200	2.50

[a] Each value represents the average of at least 3 readings taken at about 15 min intervals to allow for temperature equilibration. Readings did not fluctuate by more than ±0.000008 once the temperature had stabilised.

[bcde] As for Table 5.

[f] OD data were obtained using suitable dilutions, in I=0.05 phosphate buffer pH 7.0, of the dialysed material used for the refractive index determinations.

[g] Virus concentration = $\frac{\text{mean } \Delta n}{0.1852 \times 10^{-3}}$ mg/ml (Scheele & Lauffer, 1967).

[h] The upper and lower uncertainty limits were obtained from the duplicate Δn values by assuming that each Δn value was subject to an uncertainty of 0.000016, as it was the difference between two reading each with an uncertainty of 0.000008 (e.g. for vulgare, the upper limit to Δn was calculated to be 0.00193 + 0.000016 = 0.001946, which was equivalent to 10.51 mg/ml).

TABLE 7

Protein extinction coefficients based on refractometric concentration determinations

TMV strain	Refractive index determinations: Temp. (^{o}C)	$n_{solvent}$[a]	$n_{solution}$[a]	Δn	Protein conc.[g] (mg/ml)	Optical density characteristics[f]: OD_{251}[b]	OD_{282}[b]	$\frac{OD_{282}}{OD_{251}}$	Dilution factor	$E^{1\ mg/ml}_{282\ nm}$[e]
Vulgare	22.0	1.33293	1.33393	0.00100	5.43±0.11[h]	0.273	0.700	2.56	10	1.29
	21.8	1.33297	1.33398	0.00101						
U2	21.7	1.33297	1.33417	0.00120	6.48±0.08	0.233	0.752	3.23	10	1.16
	21.8	1.33297	1.33417	0.00120						
Cowpea	21.8	1.33297	1.33420	0.00123	6.67±0.11	0.164	0.660	4.02	10	0.99
	22.0	1.33293	1.33417	0.00124						
Y-TAMV	21.7	1.33297	1.33447	0.00150	8.13±0.11	0.149	0.427	2.87	25	1.31
	22.0	1.33293	1.33444	0.00151						

[a]As for Table 5.

[b]Light scattering was negligible in the case of TMV protein at pH 7.0.

[e] $$E^{1\ mg/ml}_{282\ nm} = \frac{OD_{282\ nm} \times \text{dilution factor}}{\text{concentration}}$$

[f]As for Table 5.

[g]Determined using the same formula as for virus concentration (Table 5).

[h]As for Table 5.

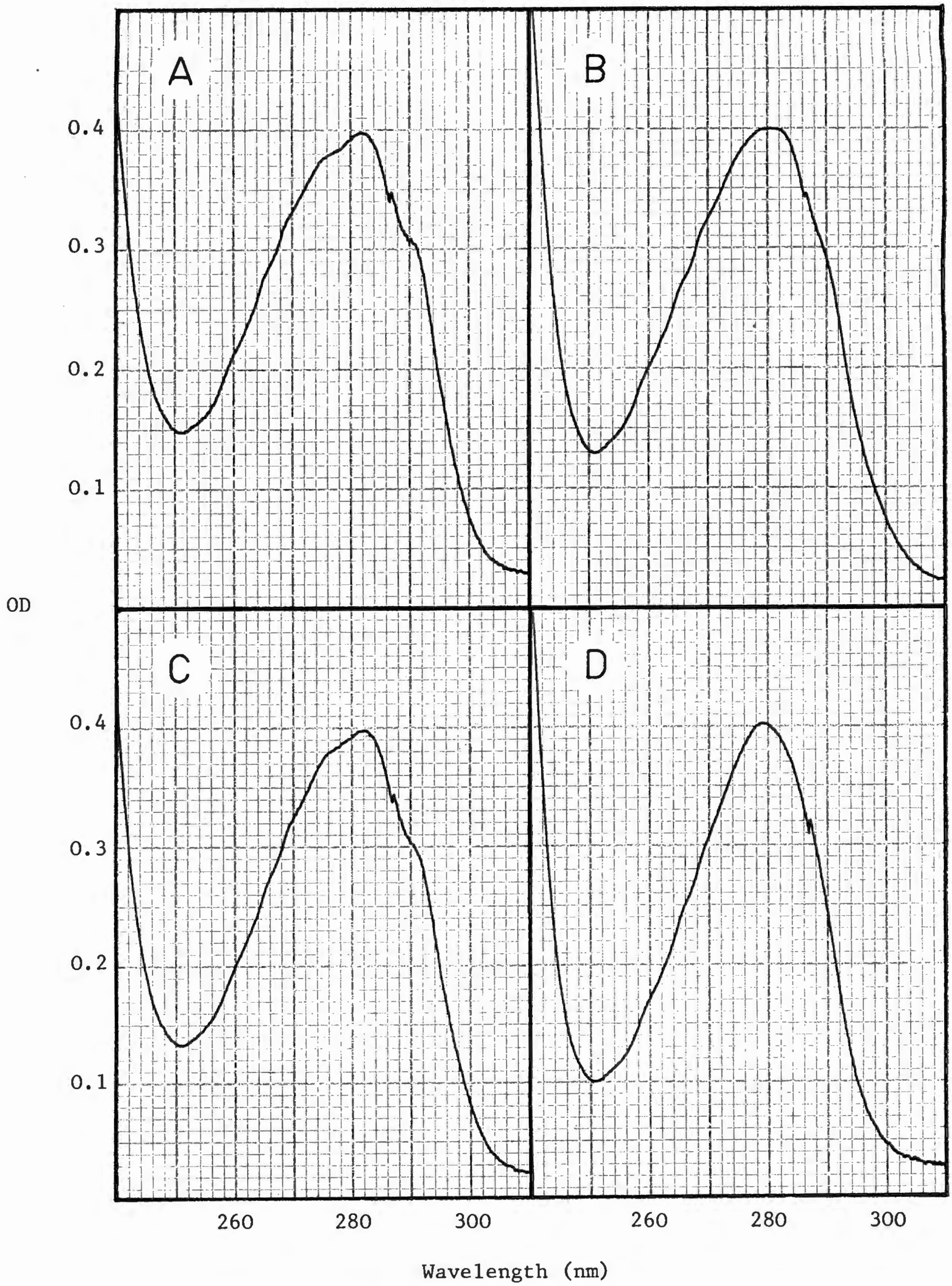


Figure 2. Ultra-violet absorbance spectra of the purified proteins of TMV type strain (A), U2 strain (B), Y-TAMV (C) and cowpea strain (D), dissolved in I=0.05 phosphate buffer pH 7.0. The protein solutions were at concentrations of about 0.31, 0.35, 0.40 and 0.30 mg/ml respectively.

The remaining residues, particularly the amounts of aspartic acid, glutamic acid, glycine, alanine and leucine, were determined to be present in amounts that showed good correspondence with the reported amino acid compositions of the four TMV strains, and justified the omission of threonine and serine from the calculation of the molar ratios. Unambiguous identification of these strains was thus possible. In particular, the single histidine and 15 leucine residues of the cowpea strain, the 22 aspartic acid and 2 methionine residues of U2, and the one methionine and 19 glutamic acid residues of Y-TAMV were evidence for these strains being distinct from vulgare and from each other.

Optical density characteristics can be used for the differentiation of the purified proteins of the four TMV strains (see Table 4 and Fig. 2). The effect of the different amounts of tyrosine and tryptophan present in the four strains can be seen in the decrease in the wavelength of maximum absorption from 282 nm in the case of vulgare and Y-TAMV to 279 nm in the case of cowpea, and in the disappearance of the tryptophan "shoulder" at 290 nm. The steady increase in OD_{max}/OD_{min} is coupled to the increase in tyrosine content because the OD_{max}/OD_{min} of tyrosine is 7.9 whereas that of tryptophan is 2.9 (Beaven & Holiday, 1952). The OD_{max}/OD_{min} figures presented in Table 3 should be regarded as minimum values, and in the present study they varied depending on <u>inter alia</u> the freshness of the protein preparation and the completeness of RNA removal. They do, however, serve to illustrate that the OD_{max}/OD_{min} figure of 2.5, reported by Durham (1972a) as indicating satisfactory removal of RNA from vulgare protein, would be an unsatisfactory criterion of purity to apply to the other three strains.

These results also demonstrate that the ethanolamine method of Durham (1972a) can be used for the preparation of protein from the Y-TAMV, U2 and cowpea strains of TMV. However, as found by Durham (1972a) with type strain, batches of U2 and cowpea strain occasionally resisted dissociation at pH 11. These batches were generally then used for the preparation of protein by the acetic acid method of Fraenkel-Conrat (1957), followed by passage through a DEAE-cellulose column to remove final traces of RNA if the OD_{282}/OD_{251} figure was below that reported in Table 4.

Extinction coefficients (E) for the four strains of TMV were determined in this study because of the large discrepancies in the values reported in the literature. Extinction coefficients ranging from 2.7 to 3.5 for a 0.1% solution have been determined at 260 nm for TMV type strain (Brakke, 1967). As the E determined for a particular virus can vary depending on the freshness and the history of the batch used for the determination (i.e. the method of purification, the degree of aggregation or degradation, and the pH and composition of the suspending medium), E values were determined using freshly purified virus (see Materials & Methods, section A2) diluted in I=0.05 phosphate buffer pH 7.0. Of the two methods of quantitation used, dry weight determination with a standard deviation of 0.3% appeared marginally more accurate than refractometry with a standard deviation of about 1%. However, the drying and weighing were tedious and time-consuming, and the accuracy was largely dependent on rigorous standardisation of the drying, handling and weighing procedure. Refractometry was uncomplicated and quick, and is the method of choice provided the temperature is controlled to within $0.1^{o}C$ and the concentration of virus or protein is sufficiently high. An uncertainty in Δn of 0.000016 corresponded to a protein concentration of 0.086 mg/ml. A protein solution being measured would thus have to have a concentration of at least 8.6 mg/ml in order to reduce the level of this uncertainty to less than 1% of the total concentration being measured.

The spectrophotometric results (Tables 5 & 6) underline the importance of the light scattering correction when quantitating virus. The plots of log OD versus log λ generally deviated little from linearity, with correlation coefficients seldom below 0.99, but had variable slopes. A λ^{-4} correction is often used as a rule of thumb to compensate for light scattering, e.g. from the OD at 320 nm the scattering at 260 nm is calculated as being equal to $OD_{320\ nm} \times \frac{320^4}{260^4}$. However, the slopes listed in Tables 5 and 6 vary from -2.8 to -4.14, indicating that for accurate quantitative work a correction factor for each preparation must be determined graphically. This correction usually resulted in a reduction of between 18% and 21% in the observed $OD_{260\ nm}$, but was occasionally as low as 14%.

The virus extinction coefficients based on dry weight determinations are within 2% of those obtained using refractometry. The value of 2.58 for the $E^{1\ mg/ml}_{260\ nm}$ of TMV vulgare is lower than the previously reported values of 2.7 (Fraenkel-Conrat, 1974), 3.01 (King & Leberman, 1973), 3.06 (Boedtker & Simmons, 1958) and 3.24 (Brakke, 1971). Values ranging from 2.7 to 3.5 (not corrected for light scattering) have appeared in the literature (Zaitlin & Israel, 1975; Brakke, 1967), and the results of the present study indicate that these values would have been about 20% lower had they been corrected. Only King & Leberman (1973) corrected their value for light scattering, by utilising the absorbance spectrum between 240 nm and 360 nm. They provided no further experimental details, except that their TMV preparations were stored at a concentration of 20 mg/ml in distilled water at 4^{o}C. Both King & Leberman (1973) and Boedtker & Simmons (1958) determined virus concentration using dry weight measurements. In view of the absence of experimental details, it is not possible to comment on the merits of each of the above determinations. Their range is probably due to both inaccuracies of measurement, and the physical differences in TMV preparations originating in different laboratories. An uncorrected value of 2.7 (Fraenkel-Conrat, 1974) would on correction for light scattering have yielded a value well below that of 2.58 obtained in the present study. Also, Titration hysteresis indicates that a rearrangement in the quaternary structure of a protein aggregate is being induced by the dissociation of one or more ionisable groups. That both the swelling and the hysteresis occur near pH 7, and that the former involve divalent cations, implicates anomalously titrating carboxyl groups that constitute a divalent cation binding site and that control the capsid stability of BMV. This implication would be confirmed if it could be demonstrated that BMV is able to bind divalent cations, and that the titration hysteresis is reduced by such binding.

Turnip crinkle virus (TCV) undergoes a rapid increase in breakdown above pH 7.8 which is further enhanced at ionic strengths above I=0.4 (Durham, 1971). These facts suggest that a group titrating near neutrality is implicated in the control of breakdown, and that RNA-protein bonds contribute to the stability of the virus. In view of the suggested role of abnormally titrating groups and of calcium-stabilised RNA-protein bonds in TMV, an investigation of cation binding by TCV was made, to establish whether a parallel situation exists in this virus.

Potentiometric titration, both in the absence and in the presence of divalent cations, was used to investigate cation binding to BMV, to TCV, and to turnip yellow mosaic virus (TYMV) which has been reported to contain substantial amounts of metal ions.

(5) The binding of cations should alter the aggregation state of tobacco mosaic virus protein.

If divalent cations have a stabilising effect on TMV, one would expect them to stabilise, or perhaps even alter, the aggregation state of TMVP. Evidence for this occurring, at least under certain circumstances, was presented by McMichael & Lauffer (1975) who reported that a TMVP aggregate produced by acidification to below pH 6 was stabilised by bound Ca^{2+} ions against a temperature-induced depolymerisation at if the uncorrected OD_{260} value of 0.633 (Table 6) had been used for the calculation of an $E_{260\ nm}^{1\ mg/ml}$ for vulgare, a value of 3.07 would have resulted, which is within the range of uncorrected values listed above.

These discrepancies serve to underline a number of facts. Firstly, a macromolecule like TMV, with tendencies towards both degradation and aggregation, cannot be assigned a single, universally-applicable extinction coefficient, either corrected or uncorrected. Secondly, for accurate quantitative work, an uncorrected extinction coefficient is of limited value because of the unpredictable variability from laboratory to laboratory in the physical properties and therefore in the degree of light scattering of the purified product. Thirdly, in view of the impracticality of determining a corrected extinction coefficient for each fresh preparation, purification procedures in a laboratory should be standardised and, for routine quantitation, an accurate corrected extinction coefficient should be determined using a representative sample. This was the procedure adopted in this study, and its use was subsequently justified by the reproducibility from batch to batch of the potentiometric titration results.

B. TMV type strain

1. Virus dissociation in vitro

(a) Assay method for dissociated protein

The Lowry protein assay was the original method of choice, and was found with TMV protein in dilute phosphate buffer to give a linear calibration plot over the range 5 to 200 µg/ml using 1 ml samples. However, both Ca^{2+} and EDTA were found to interfere with the Folin reagents used in this method. The former produced a precipitate at Ca^{2+} concentrations higher than 1.25 mM, while the latter caused a spurious colour reaction. The presence of 10 mM EDTA resulted in an OD equivalent to that produced by 60 µg protein/ml. Addition of sucrose, however, reduced this EDTA effect, such that 10 mM EDTA plus 15% sucrose resulted in an OD equivalent to only 10 µg protein/ml. The presence of sucrose alone, up to a concentration of 30%, interfered negligibly with the Lowry test. This method of assay was thus avoided.

The ninhydrin protein assay yielded linear calibration plots using 0.5 ml samples over the range 5 to 160 µg protein/ml, i.e. 2.5 to 80 µg total mass. Both Ca^{2+} and EDTA interfered very little, although phosphate ions resulted in a precipitate which raised the OD. This latter observation may account for the fact that calibration curves using TMV yielded OD's nearly double those obtained using TMVP over the same concentration range. The points on the ninhydrin calibration plots tended to show more scatter than in the case of the Lowry method, and the slopes varied depending on the batch and age of the reagents. The ninhydrin method was subsequently used for all protein assays.

(b) Attempts at virus dissociation

(i) Effect of Ca^{2+} concentration

After 48 h incubation of TMV (type strain) at 4°C in 10 mM EDTA buffers at pH 7.0 and I=0.1, followed by centrifugation in the SW 27.1 rotor at 135 000 g for 180 min at 4°C, very little protein was detectable in the supernatants of either the pCa 9 or the pCa 3 solutions. The former supernatants appeared to contain slightly more protein than the latter supernatants (see Table 8).

TABLE 8

Effect of Ca^{2+} concentration on TMV dissociation

(pH 7.0, I=0.1, 4°C, 10 mM EDTA)

Sample	OD_{570} 1	OD_{570} 2	OD_{570} Ave.	OD_{570} Corrected[a]	Protein concentration (μg/ml)	% of original sample
Blanks						
10 mM EDTA pCa 9	0.078	0.083	0.079			
10 mM EDTA pCa 7	0.087	0.080				
10 mM EDTA pCa 3	0.072	0.070				
pCa 9 supernatants						
10 mg TMV[b]	0.116	0.115	0.116	0.037	3.0	0.030
20 mg TMV	0.131	0.133	0.132	0.053	6.6	0.033
40 mg TMV	0.159	0.172	0.166	0.087	13.8	0.035
pCa 3 supernatants						
10 mg TMV[b]	0.080	0.072	0.076	0	<1	0
20 mg TMV	0.075	0.073	0.074	0	<1	0
40 mg TMV	0.078	0.077	0.078	0	<1	0
Calibration[c]						
5 μg TMV/ml	0.134	0.124	0.129	0.050		
25 μg TMV/ml	0.227	0.226	0.227	0.148		
50 μg TMV/ml	0.330	0.336	0.333	0.254		
75 μg TMV/ml	0.457	0.427	0.442	0.363		
100 μg TMV/ml	0.595	0.562	0.579	0.500		
125 μg TMV/ml	0.735	0.715	0.725	0.646		

[a]Corrected OD_{570} = average OD_{570} of sample minus average OD_{570} of blanks.

[b]Figures represent the amount of TMV per 17 ml centrifuge tube prior to centrifugation.

[c]For calibration, TMV was diluted in 10 mM EDTA pCa 7.

(ii) Effect of pH

As removal of Ca^{2+} at pH 7.0 did not result in appreciable virus dissociation, the effect of Ca^{2+} removal on TMV (type strain) was examined at different pH's ranging from 7.0 to 9.0. After 24 h incubation of 20 mg aliquots of TMV at $4^{o}C$ in 10 mM EDTA buffers at I=0.1, followed by centrifugation at 135 000 g for 3 h, dissociated protein was detectable in the supernatants at pH's above 7.5. The amount of dissociated protein increased with increasing pH (see Figure 3), such that at pH 9.0 nearly 20% of the starting material was present in the supernatant as dissociated products. The values plotted in Figure 3 were obtained in a manner analogous to that used in Table 8.

(iii) Effect of ionic strength, EDTA concentration and TMV concentration

In order to establish whether lowering the ionic strength would induce virus dissociation upon removal of Ca^{2+} at pH 7.0, 1.0 mM EDTA solutions at pH 7.0 were adjusted with KCl to different ionic strengths ranging from I=0.004 to I=0.1 (Table 1). Type strain of TMV was dialysed against a large excess of 1 mM KCl at $4^{o}C$. Aliquots containing 1.2 mg TMV/ml were dialysed against the various EDTA solutions for 48 h, one being left in 1 mM KCl as control. The supernatants were then assayed for protein after centrifugation at 135 000 g for 3 h (see Table 9).

The effect on virus dissociation of lowering the virus concentration followed by removal of Ca^{2+} was examined by dialysing different concentrations of TMV, ranging from 0.1 to 3.2 mg/ml, against 10 mM EDTA pH 7.25 I=0.1. In a similar experiment aliquots of TMV at a concentration of 1.2 mg/ml were dialysed against solutions containing different concentrations of EDTA ranging from 5 to 50 mM, to examine the effect of raising the EDTA concentration. The ionic strength was in each case adjusted to I=0.25 with the appropriate

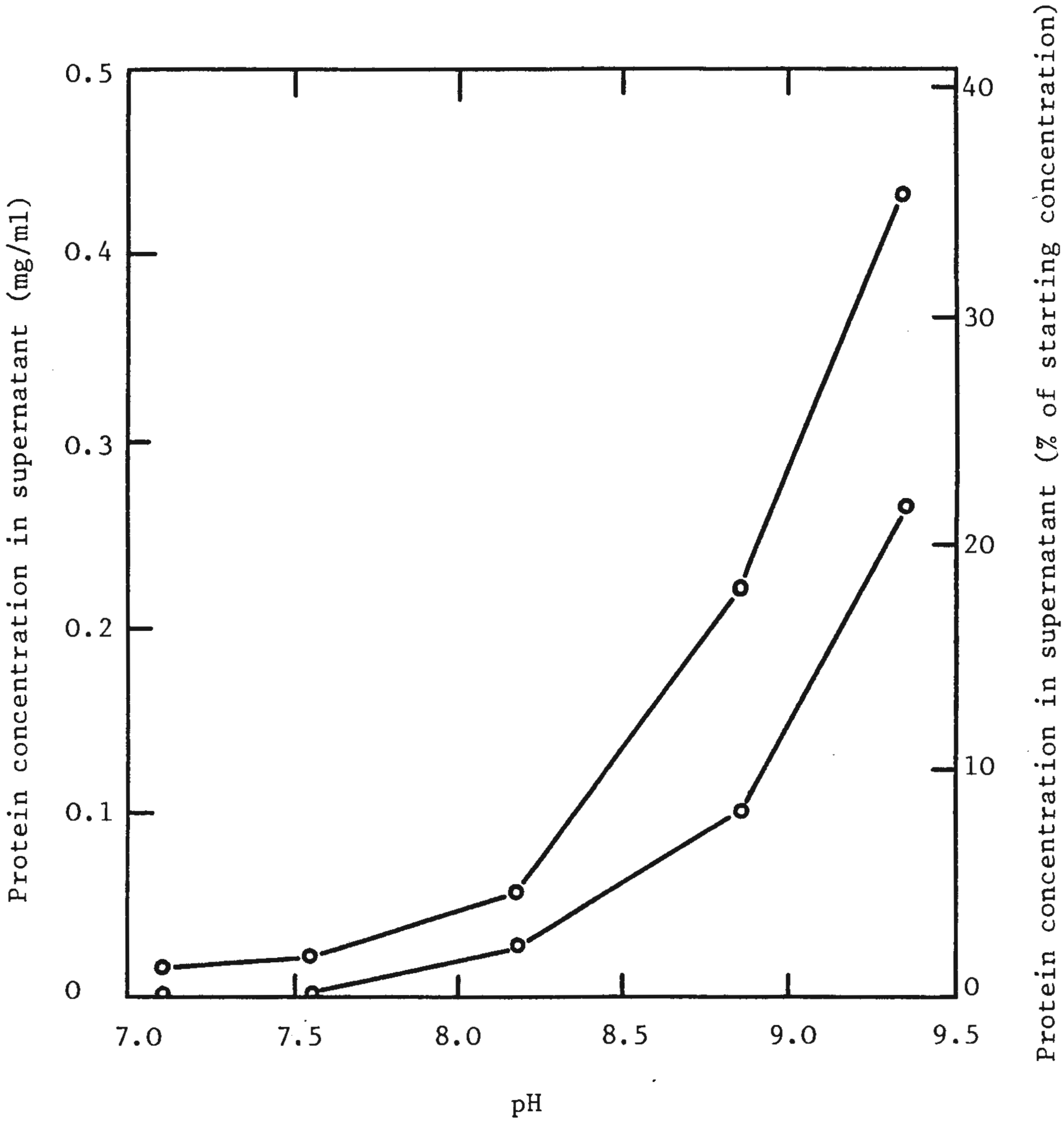


Figure 3. Dissociation of TMV type strain as a function of pH. Aliquots of 17 ml, each containing 20 mg of TMV in 10 mM EDTA at I=0.1, were adjusted to pH's ranging from 7.1 to 9.4, incubated at $4^{o}C$ for 24 h, and spun at 135 000 g for 3 h. Dissociated protein was monitored in the top 2 ml of supernatant (lower curve) and in the rest of the supernatant (upper curve) by means of the ninhydrin protein assay.

amount of KCl. After 96 h dialysis at 4°C, the preparations were centrifuged and the supernatants assayed for protein (see Table 10).

TABLE 9

The effect of ionic strength on the production of non-sedimentable TMVP

(1 mM EDTA, pH 7.0, 4°C, 1.2 mg TMV/ml)

Ionic strength (I)	Contribution of KCl to final ionic strength[a]	Protein in supernatant (μg/ml)[b]	% of original protein conc. in supernatant
0.004	-	4.4	0.4
0.005	0.001	6.8	0.6
0.01	0.006	7.2	0.6
0.05	0.046	<1	<0.1
0.10	0.096	9.0	0.7
0.001 (control)	0.001	12.6	1.0

[a] I of 1 mM EDTA at pH 7.0 = 0.004

[b] Determined in a manner analogous to that of Table 8.

TABLE 10

The effect of virus and EDTA concentrations on the production of non-sedimentable TMVP (pH 7.25, 4°C)

Virus concentration (mg/ml)	EDTA concentration (mM)	Ionic strength (I)[a]	Protein in supernatant (μg/ml)	% of original protein conc. in supernatant
0.1	10	0.1	<1	<0.1
0.2	10	0.1	<1	<0.1
0.4	10	0.1	<1	<0.1
0.8	10	0.1	<1	<0.1
1.6	10	0.1	<1	<0.1
3.2	10	0.1	<1	<0.1
1.2	5	0.25	<1	<0.1
1.2	10	0.25	<1	<0.1
1.2	15	0.25	1	0.1
1.2	30	0.25	2.5	0.2
1.2	50	0.25	2.5	0.2
1.2	0	0.001	<1	<0.1

[a]Ionic strengths were adjusted by the addition of KCl.

(c) Discussion

The method of Lowry *et al.* (1951) for protein quantitation has the advantages of simplicity and reproducibility, although certain substances are known to interfere with the assay (Bonitati *et al.*, 1969, and the references listed therein). The more intricate ninhydrin assay was used in this study because both Ca^{2+} and EDTA were found to interfere with the Folin reagents.

Due to the many steps involved in the ninhydrin assay method, this assay was found to be less reproducible than that of Lowry et al. (1951), but was marginally more sensitive. Also, the presence of phosphate ions caused a rise in the OD_{570} of the final reaction mixture, thereby casting doubt on the accuracy of ninhydrin protein assays done in the presence of RNA. However, this phenomenon would have served to enhance the detection of virus dissociation, as both the liberated RNA and protein would have been present in the final supernatant. When using the ninhydrin method of protein assay, it is possible to detect 2 μg TMV/ml. Therefore, in a suspension of TMV of concentration 2 mg/ml, dissociation to the extent of 0.1% may be detected.

It is unlikely that the low level of protein concentration observed in supernatants was due to depletion as a result of sedimentation of the dissociated protein. The pH was always maintained at 7.0 or higher, and the ionic strength kept below I=0.25, under which conditions the predominant protein aggregates would have been one entity sedimenting at 4 S and another sedimenting at 20 S (Durham, 1972a). At 135 000 g there would have been negligible sedimentation of the 4 S protein species, while the species of 20 S would have required 6 h to sediment half-way down the tube. Also, under conditions where dissociation occurred (Figure 3), the protein concentration in the top 2 ml of the supernatant was of the same order of magnitude as that in the bottom region. The progressive increase in detectable protein near the meniscus with increase in pH above 7.5 could not have been due to the higher pH which favoured formation of the 4 S aggregate to an increasing degree at the expense of the larger 20-30 S species. At pH 7.25 and above, the equilibrium favours dissociation to the extent that no aggregates larger than 4-7 S are detectable in the solution (Durham, 1972a).

Removal of divalent cations induced minimal virus dissociation under approximately physiological conditions of pH (7.0-7.5) and ionic strength (I=0.1-0.25). Little dissociation occurred if the ionic strength was lowered to I=0.004, the EDTA concentration was raised to 50 mM, or the virus concentration varied between 0.1 and 3.2 mg/ml. However, above pH 7.5 progressively more dissociation occurred as the pH was raised, until at pH 9.0 about 40% of the starting material was present in the supernatant. Alkaline dissociation of TMV has been well documented (Wyckoff, 1937; Schramm _et al._, 1955; Harrington & Schachman, 1956; Caspar, 1963; Diener & Desjardins, 1966; Perham, 1969; Brakke & van Pelt, 1969; Onda _et al._, 1970; Brakke, 1971; Powell, 1975; Perham & Wilson, 1976), although most of these investigations were performed in the region of pH 10. However, preparations of TMV are known to contain variable amounts of particles that are totally resistant to degradation at pH 10, while sensitive particles degrade predominantly to particles 100 nm in length (Schramm _et al._, 1955; Harrington & Schachman, 1956; Perham, 1969).

Reports differ as to the pH at which alkaline degradation of TMV commences. Harrington & Schachman (1956), using an analytical ultracentrifuge with schlieren optics to follow virus dissociation, reported that TMV was stable at pH 8.6. However, it is unlikely that they would have detected less than 10% dissociation, 5-10% being the extent of dissociation found at pH 8.6 in this study. Brakke & van Pelt (1969) using the considerably more sensitive technique of density gradient centrifugation followed by scanning of the gradient column at 254 nm, detected TMV degradation in 0.02 M tris: 0.001 M EDTA buffers at pH 8.0, but not at pH 7.5. The failure in the present study to induce virus dissociation by cation removal at or near neutrality is thus in accordance with the findings of Brakke & van Pelt (1969). It also agrees with the findings of R.L. Steere, mentioned in the same publication, that TMV preparations had remained monodisperse for over a year in dilute EDTA at pH 7.5. Alkaline dissociation of TMV in buffers of I=0.004 to 0.25 is thus insignificant at pH 7.5, although it is detectable at pH 8.0.

The role of divalent cations in the alkaline degradation of TMV is obscure. This study has not established whether cation removal was a contributory factor to the dissociation observed, although Brakke & van Pelt (1969) found that degradation at pH 9.0 could be reduced, but not prevented, by 10^{-2} to 10^{-4}M Mg^{2+}. The cations affected the *in vitro* separation of protein from nucleic acid (Brakke & van Pelt, 1969; Brakke, 1971). Powell (1975) reported that cations protected against complete dissociation by stopping the stripping process at certain sites along the nucleic acid. The author stated that his data could best be reconciled with a mechanism whereby the cation linked the protein to the nucleic acid, perhaps via the carboxyl group of an acidic amino acid and a nitrogen from a purine or pyrimidine ring. Whatever the function of these cations, their presence is not mandatory for the stability of the TMV particle at neutral pH.

In view of the inability in the present study to induce *in vitro* TMV dissassembly by divalent cation removal near neutral pH, it was apparent that the first prediction of section D in Chapter 2 was too simplistic. Disassembly of TMV *in vivo* was thus unlikely to be due solely to the low calcium concentration of the cytoplasm, various other undefined intracellular factors being required. It was thus decided further to characterise divalent cation binding by TMV and various other plant viruses in order to verify the remaining predictions mentioned in section D, Chapter 2, and to attempt to clarify the well-documented effects that divalent cations have on the structure or stability of these viruses.

2. Direct measurement of bound calcium (to all four TMV strains)

A knowledge of the extent of calcium binding by TMV under various conditions of pH and pCa was an essential prerequisite to any investigation of the role of bound calcium ions. Calcium binding to TMV was thus quantitated by equilibrium dialysis and by a sedimentation method.

(a) Equilibrium dialysis

The results of the calcium ion equilibrium dialysis experiments on vulgare are given in Figure 4. These results were obtained at ionic strength I=0.1, and it is evident that the number of Ca^{2+} ions bound increases as the pH increases, or as the pCa decreases. The dotted line indicates the pH and pCa values at which approximately 1 Ca^{2+} ion was bound per protein subunit. At pCa values of 2.7 and 3.0, the number of calcium ions bound was found to decrease as the pH increased above pH 8.0. Very little calcium binding occurred below pH 6.0 over the pCa range investigated. The maximum binding of calcium occurred at pH 7.8 where, at a pCa value of 2.7, two calcium ions were bound per protein subunit.

(b) Determination of bound calcium by sedimentation

The amounts of calcium bound by the four TMV strains, as determined by the sedimentation technique, are illustrated in Figure 5 and summarised in Table 11.

TABLE 11

The binding of calcium to four TMV strains at different pH and pCa values as measured by the sedimentation method

		Ca^{2+} ions bound per protein subunit[a]			
pCa	pH	Vulgare	Y-TAMV	U2	Cowpea
3.0	7.0	0.66	0.77	1.35	0.84
2.7	6.0	0.54	0.74	1.40	0.32
2.7	7.0	0.91	1.11	1.54	1.20
2.7	8.0	1.00	1.80	1.66	2.24

[a] See Appendix 2 for a specimen calculation.

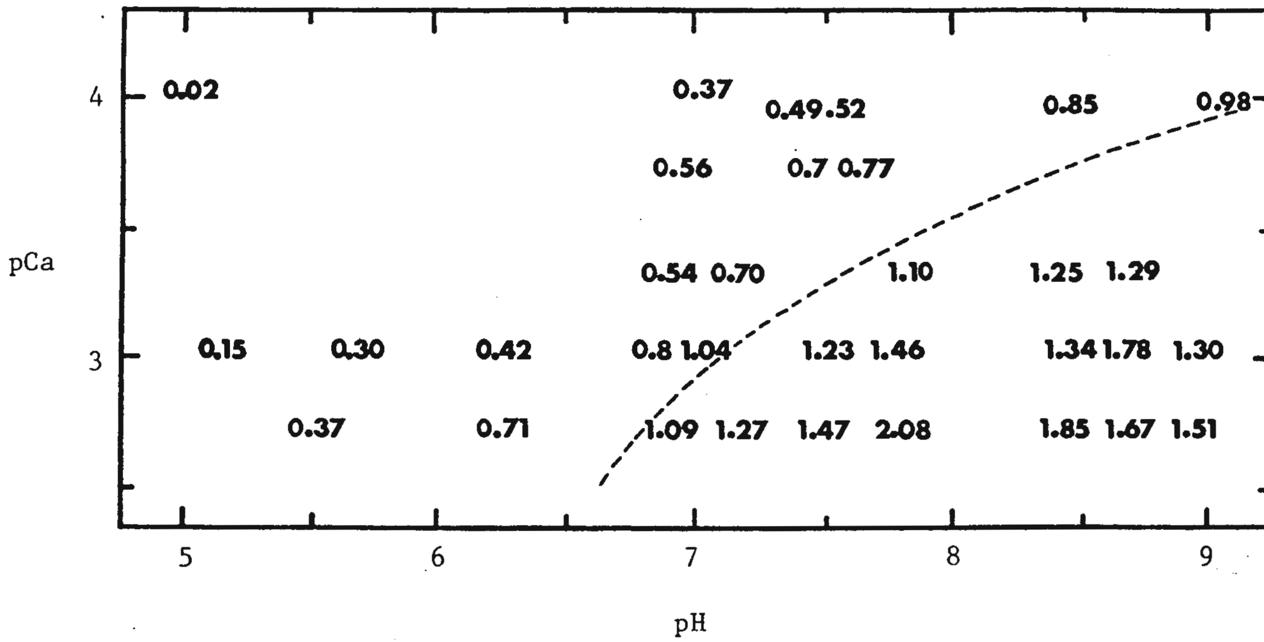


Figure 4. The number of Ca^{2+} ions bound per protein subunit to TMV vulgare, as a function of pH and pCa. The precise pH and pCa for each number is indicated by the position of the decimal point. Scintillation counting was used to measure the binding of ^{45}Ca to TMV, after equilibrium dialysis at 6°C in buffers of ionic strength I=0.1. The dotted line indicates the conditions of pH and pCa under which a net total of approximately one Ca^{2+} ion is bound per TMVP subunit.

The plots of change in calcium concentration versus virus concentration showed little deviation from linearity (least-square regressional analyses indicated that only two plots had correlation coefficients of less than 0.99), but the majority did not pass through the origin. The linearity of the plots indicated that calcium removal was proportional to virus concentration under the experimental conditions employed.

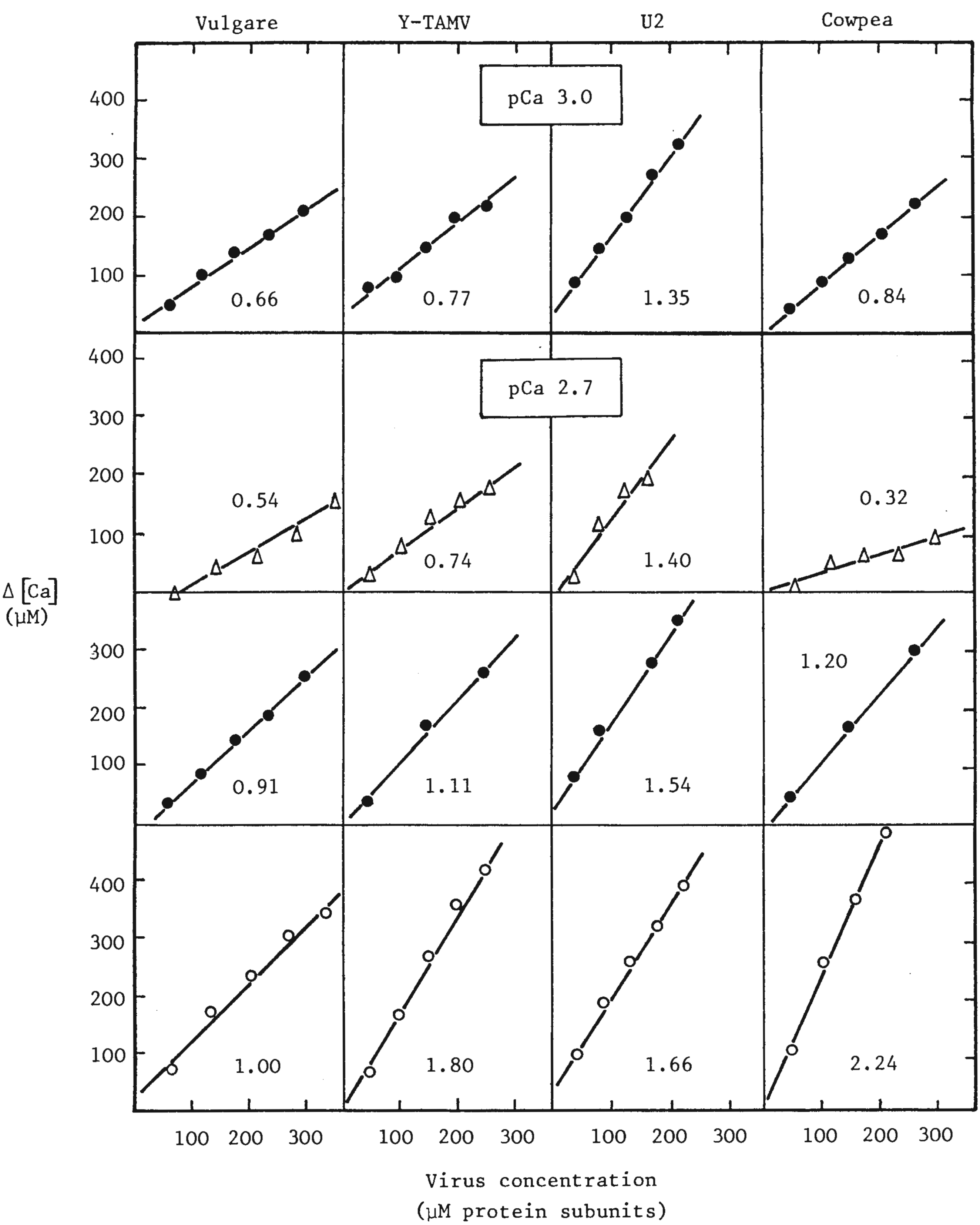


Figure 5. The binding of calcium to four strains of TMV. The points represent the reduction in calcium concentration (i.e. the calcium concentration before centrifugation minus the calcium concentration in the supernatant after centrifugation, determined by scintillation counting) in virus solutions at different virus concentrations, after the virus particles plus bound calcium had been removed by centrifugation at 40 000 r.p.m. for 2 h. Prior to centrifugation, the virus solutions at pH 6.0 (triangles), pH 7.0 (closed circles) or pH 8.0 (open circles) were adjusted to either pCa 3.0 (upper four plots) or pCa 2.7 (lower twelve plots) by the addition of 10 mM $CaCl_2$ containing ^{45}Ca. The figures accompanying each plot are the slopes of the regression lines, and are equivalent to calcium ions bound per protein subunit.

The slopes of the plots in Fig. 5 yielded the values of Ca^{2+} bound per subunit which are listed in Table 11. It is evident here, as with the equilibrium dialysis results, that calcium binding increases as the pH increases, and as the pCa decreases. The type strain was consistently the weakest binder of calcium, followed by Y-TAMV. The U2 strain appeared to be the strongest binder of calcium, except at pH 8.0 and pCa 2.7 where cowpea strain bound more than two Ca^{2+} ions per subunit. However, the binding of calcium to the cowpea strain appeared more sensitive to pH than was the case with the three other strains, 1.20 Ca^{2+} ions being bound per subunit at pH 7.0 and only 0.32 at pH 6.0. The binding of calcium by U2 was the least sensitive to pH, the number of Ca^{2+} ions bound per subunit decreasing from 1.66 at pH 8.0 to 1.40 at pH 6.0. The binding of calcium by vulgare was similar to the binding by Y-TAMV, except at pH 8.0 where Y-TAMV bound nearly twice as much calcium as did vulgare. Halving the calcium concentration at pH 7.0 (i.e. changing from pCa 2.7 to pCa 3.0) resulted in a roughly 25% reduction in calcium bound by all the four strains of TMV.

(c) Discussion

The equilibrium dialysis and sedimentation analysis results confirm that TMV is capable, between pH 6 and pH 9, of binding detectable amounts of calcium, as discussed in section A1(c) of Chapter 2. As the pH increases, the number of Ca^{2+} ions bound per subunit increases, due to diminished competition from protons for the binding sites (Gurd & Wilcox, 1956), and reaches a maximum near pH 8. The equilibrium dialysis results indicate that the binding decreases above pH 8.0, perhaps due to dissociation of the virus particles which is known to commence above pH 7.5 (Brakke & van Pelt,1969; also section B1(a) of this chapter). Very little binding of calcium occurred below pH 6.0, as was also found by Shalaby et al. (1968) and by McMichael (1973).

The results of equilibrium dialysis and of sedimentation agreed on the extent of calcium binding by vulgare at pH 6.0 and at pH 7.0, both at pCa 2.7. Both results were somewhat lower than the values of 0.8 Ca^{2+} ions bound per subunit at pH 6.0 and 1.3 bound at pH 6.8 (see Table 12) reported by Shalaby et al. (1968), who worked at pCa 2.7 using a potentiometric method employing ion-exchange membrane electrodes. However, these authors did not work at constant ionic strength, which in their case was generally below I=0.05.

Poor agreement between the results of the two techniques was obtained if the pH was raised to 8.0. At pH 8.0 and pCa 2.7, the sedimentation method indicated only 50% of the binding found by equilibrium dialysis at pH 7.8. The results of the latter technique were in good agreement, however, with those of Shalaby et al. (1968), who reported that 1.8 Ca^{2+} ions were bound per subunit at pCa 2.6 and pH 8.2. These authors also reported that 1.8 Ca^{2+} ions were bound per subunit at pH 9.0 and pCa 2.5, which was in reasonable agreement with the equilibrium dialysis result at pH 8.9. These results are grouped for comparison in Table 12.

In view of the reasonable agreement between the results of Shalaby et al. (1968) and those obtained by equilibrium dialysis, as well as the sound thermodynamic basis of the latter technique (see e.g., Steinhardt & Reynolds, 1969), the low values obtained by sedimentation (especially at high pH) must be viewed with scepticism. Although the ionic strength (I=0.16) of the centrifuged solutions should have been sufficient to prevent electrostatic effects, it is conceivable that some redistribution of Ca^{2+} ions might have occurred during sedimentation at the higher pH values. Alternatively, compacting the highly charged virus particles into a pellet at pH 8 might have resulted in an anomalous release of bound calcium. It is unlikely

TABLE 12

The binding of calcium to TMV vulgare at different pH and pCa values

		Ca^{2+} ions bound per protein subunit		
		Method of determination		
pH	pCa	Equilibrium dialysis	Sedimentation	Potentiometry[a]
6.0	2.7	-	0.54	0.8
6.2	2.7	0.71	-	-
6.8	2.7	1.09	-	1.3
7.0	2.7	-	0.91	-
7.0	3.0	1.04	0.66	-
7.4	2.7	1.47	-	1.4
7.8	3.0	1.46	-	-
7.8	2.7	2.08	-	-
8.0	2.7	-	1.00	-
8.2	2.6	-	-	1.8
8.4	2.7	1.85	-	1.9
8.9	2.7	1.51	-	-
9.0	2.5	-	-	1.8

[a]From Shalaby et al. (1968).

that the low values were due to alkaline degradation resulting in reduced binding, as this would have been reflected in the equilibrium dialysis and potentiometric results as well. Also, even if alkaline degradation had generated non-sedimenting subunits, these have been shown (see section D1 of this chapter; McMichael, 1973; and Shalaby et al., 1968) not to bind significant amounts of calcium, and thus could not have raised the calcium levels of the supernatants.

The plots of $\Delta[Ca]$ vs. virus concentration (Fig. 5) did not deviate from linearity, even though the free calcium concentration must have decreased from point to point in a particular plot, due to a constant amount of calcium having been added to increasing amounts of virus. This method obviously precluded the use of a calcium buffer, as calcium determinations were done on the supernatant and not on the washed pellet as originally done by Cohen & Selinger (1969). The failure of many of the plots in Fig. 5 to pass through the origin can probably be ascribed to slight discrepancies between the actual calcium concentrations in the reference blanks, and their assumed calcium concentrations used for C_o.

TMV vulgare thus binds a maximum of two Ca^{2+} ions per protein subunit near pH 8 at a calcium concentration of about 2×10^{-3} M. Shalaby et al. (1968) reported that raising the calcium concentration above about 3×10^{-3} M did not result in further binding. Due to the lack of published data, and to the above-mentioned uncertainties regarding the sedimentation technique, the situation regarding calcium binding by the other three strains is less clear. One can deduce from Fig. 5 that Y-TAMV probably binds calcium very similarly to vulgare, as would be expected from their similar amino acid compositions (Table 3). The U2 and cowpea strains bind calcium more strongly than vulgare, the cowpea strain possibly binding as many as three Ca^{2+} ions per protein subunit (see also Fig. 24 and section C1 of this chapter). However, the binding of calcium by the cowpea strain drops off rapidly as the pH is reduced, while that of U2 is only slightly affected by increased acidity.

The second prediction of section D in Chapter 2 has thus been substantiated, insofar as four strains of TMV have been shown to bind at least two Ca^{2+} ions per TMVP subunit under specified conditions of pH, ionic strength and pCa. Although the affinity of this binding has not been established, nor the number of binding sites per TMVP subunit, the overall dissociation constant must be less than 10^{-4} M for binding to have occurred at a calcium concentration of 10^{-4}M.

During the course of the above experiments, it became apparent that the technique of potentiometric titration was both more convenient and more informative, as well as less time-consuming, than equilibrium dialysis. This latter technique was thus discontinued in favour of hydrogen-ion titration. Information concerning the number of binding sites and their affinities for cations, which was obtained by titration, will be presented in the next section of this chapter.

3. Titration of tobacco mosaic virus

In the previous section, it was established that four TMV strains are each able to bind at least two calcium ions per protein subunit. As calcium is thought to bind to, and displace protons from, binding sites containing anomalously-titrating carboxyl groups (Durham & Butler, 1975), cation binding to TMV was investigated by comparing the potentiometric titration behaviour of TMV in the absence and in the presence of various concentrations of multivalent cations.

(a) Confirmation of the removal of bound cations prior to titration

For the titration of TMV in the absence of divalent cations, it was essential to confirm that dialysis of TMV against EDTA was removing cations quantitatively. Preparations of TMV that had been dialysed for 24 h at 4°C against 50 mM EDTA at pH 7.5, followed by dialysis against 50 mM KCl, contained negligible amounts of Ca^{2+} or Mg^{2+}, as determined by atomic absorption spectroscopy (Table 13). Prior to the dialysis against EDTA, purified TMV in 50 mM KCl contained significant amounts of these cations, while low cation levels (comparable to those obtained after dialysis against EDTA) were obtained if purified virus was dialysed at 4°C for 48 h against excess 50 mM KCl only. The TMV preparations were thus regarded, for titration purposes, as being free of divalent cations. The concentrations of other divalent cations were not determined, as these (mainly Zn, Al, Fe and Mn) have been reported to be bound by TMV to a lesser extent than are Ca^{2+} or Mg^{2+} (Loring et al., 1962; Wacker et al., 1963).

(b) Titration of TMV type strain

The acid-base titration of divalent cation-free TMV vulgare was repeated on separate batches of TMV, and the curve found to be reproducible (curve a, Fig. 6A). Forward curves obtained using acid titrant were identical to the reverse curves obtained using alkali titrant, indicating the absence of hysteresis. A total of 5.0 protons per subunit titrated between pH 4.5 and pH 8.5. Above curve (a), accurately positioned vertically to illustrate the relative degrees of protonation, are the titration curves obtained in the presence of 1.5 mM calcium (curve b, Fig. 6A) and 27 mM calcium (curve c, Fig. 6A). The positioning of curves (b) and (c) above curve (a) indicates that, at every pH between 4.5 and 8.5, protons are displaceable from TMV by calcium. The displacement of these protons at the two calcium concentrations is represented as a function of pH by the differential curves in Fig. 6B. Proton displacement by calcium is at a minimum at both extremes of pH, but reaches maxima of about 1.1 protons per subunit at pH 7.3 in the case when 1.5 mM calcium was added (curve a, Fig. 6B), and about 1.6 protons per subunit at pH 5.5 in the case when 27 mM calcium was added (curve b, Fig. 6B). Curve (b), in addition, shows a second maximum at pH 7.2.

TABLE 13

Removal by dialysis of calcium and magnesium bound to TMV

Sample[a]	Strain of TMV	Calcium determinations[b]					Magnesium determinations[b]				
		Virus conc.		Ca^{2+} conc.		Ca^{2+} ions per subunit	Virus conc.		Mg^{2+} conc.		Mg^{2+} ions per subunit
		mg/ml	µM TMVP[c]	mg/ 1[d]	µM[e]		mg/ml	µM TMVP[c]	mg/1[d]	µM[e]	
Twice-distilled water				0.4					<0.05		
50 mM KCl				0.4					<0.05		
Virus prior to dialysis vs. EDTA	vulgare	4.3	234	2.2	44.1	0.19	5.0	272	0.1	4.2	0.02
	U2	4.9	267	16.0	382.4	1.43	5.0	272	0.3	12.6	0.05
	cowpea	4.6	252	7.7	178.9	0.71	-	-	-	-	-
	Y-TAMV	5.1	277	8.0	186.3	0.67	5.0	272	0.8	33.6	0.12
Virus after dialysis vs. 50 mM EDTA	vulgare	5.3	288	0.5	2.5	0.01	3.0	163	<0.05	<2.1	<0.01
	U2	2.5	140	0.5	2.5	0.02	3.0	163	<0.05	<2.1	<0.01
	cowpea	6.4	348	0.5	2.5	0.01	3.0	163	<0.05	<2.1	<0.01
	Y-TAMV	4.9	268	0.5	2.5	0.01	3.0	163	<0.05	<2.1	<0.01
Virus after dialysis vs. 50 mM KCl	vulgare	4.3	234	0.5	2.5	0.01	5.0	273	0.1	4.2	0.02
	U2	4.9	268	0.5	2.5	0.01	5.0	272	0.1	4.2	0.02
	cowpea	4.6	252	0.5	2.5	0.01	-	-	-	-	-
	Y-TAMV	5.1	277	0.3	<2.5	<0.01	5.0	272	0.1	4.2	0.02

[a]The solvent for all the virus samples was 50 mM KCl.

[b]Determined by atomic absorption spectroscopy using a N_2O/C_2H_2 flame and lamps emitting at 285.2 nm for Mg and 422.7 nm for Ca.

[c]Calculated assuming an RNA content of 5% for TMV and a molecular weight of 17 500 for TMVP.

[d]Read off calibration plots compiled using 50 mM KCl samples to which known amounts of the cation had been added. Known amounts of cation were also added to TMV samples (that had been dialysed against EDTA) to test for possible interference by the nucleoprotein; interference was found to be negligible.

[e]Calculated from the figures in mg/l after these had been corrected for the blank readings.

The titration curves for TMV in the presence of magnesium are shown in Fig. 7A. Magnesium displaces protons from TMV over the entire pH range 4.5 to 8.5. A concentration of 1.5 mM magnesium (curve a, Fig. 7B) resulted in a maximum displacement of about 0.9 protons per subunit at pH 7.4, while 27 mM magnesium (curve b, Fig. 7B) displaced a maximum of about 1.4 protons per subunit at pH 6.9. Magnesium did not displace protons strongly at pH 5.5 although this was the pH at which maximum proton displacement by calcium was observed.

Proton displacement from TMV by strontium (Figs 8A and 8B), barium (Figs 9A & 9B) and manganese (Figs 10A & 10B) at 1.5 mM final metal concentrations all showed a similar pattern to that by magnesium and calcium. The curves of proton displacement each showed a maximum near pH 7.3, and the number of protons displaced per subunit lay between 0.9 and 1.3. At 27 mM concentrations of the metals, the proton displacements by barium, strontium and magnesium were very similar, with maxima of 1.3 to 1.4 protons displaced per subunit near pH 6.8. Manganese at a final concentration of 27 mM displaced protons very strongly, 2.1 protons per subunit being displaced at pH 6.6. Neither strontium, barium nor manganese showed maxima of proton displacement near pH 5.5, this feature being peculiar to proton displacement by calcium.

The patterns of proton displacement by lead (Figs 11A & 11B) and by lanthanum (Figs 12A & 12B) were markedly different from those of the five afore-mentioned cations. Saturating amounts of lead could not be added, because the hydrated lead ion acts as a proton donor above pH 6 (Gurd & Wilcox, 1956). Titrations were done in the presence of lead concentrations equivalent to ½, 1, 2 and 3 Pb^{2+} ions per subunit, and in each case maximum proton displacement occurred near pH 6.0. Except at the highest lead concentration, the addition of Pb^{2+} ions resulted in the displacement of more than a stoichiometric amount of H^+. Lanthanum, a trivalent cation, was a powerful displacer of protons. At a 1.5 mM concentration of La^{3+}, a maximum of 2.4 protons per subunit was displaced at pH 5.9. Titrations in the presence of higher La^{3+} concentrations were not attempted; upon the addition of 100 µl of 100 mM La^{3+}, the virus solution went milky white and did not clear during the titration.

The differential curves illustrating proton displacement from type strain by the various cations are combined in Fig. 13 for comparison.

(c) Discussion

(i) Technical considerations

The results of atomic absorption spectroscopy (Table 12) indicated that dialysis of TMV against 50 mM EDTA at pH 7.5 resulted in virtually complete removal of both bound Ca^{2+} and Mg^{2+} ions. This was in contrast to the findings of Wacker et al. (1963) who reported that a certain proportion of the calcium and magnesium bound to TMV, apparently complexed to a binding site involving the RNA, could not be removed by dialysis against EDTA. However, it is conceivable that in their hands cations could have been reacquired by the virus during the subsequent dialysis step performed to remove the EDTA. These could have been leached from glass containers, particularly as no other positively charged counter-ions were included in the water used for this dialysis.

The low metal content of the TMV strains after dialysis against 50 mM KCl only was an unexpected result. However, as will be discussed below, the class of binding sites on TMV vulgare with the highest affinity for calcium has a pK_{Ca} of 5. Thus, for at least 50% dissociation of Ca^{2+} from these sites to have occurred, in addition to virtually complete dissociation from the other sites with weaker affinities, the 50 mM KCl solution would have had to have a pCa of at least 5. This was, in fact, the calcium level in the 50 mM KCl solutions as determined by atomic absorption spectroscopy (Table 12). All KCl solutions were routinely prepared by dissolving highest purity Merck KCl in quartz-distilled deionised water, and were kept in polyethylene containers.

The type strain of TMV did not show the hysteresis between forward and reverse titrations observed by Ansevin et al. (1964). This absence of hysteresis was observed on at least five separate batches of virus, and as Ansevin et al. (1964) made no mention of having checked their virus preparations for the presence of dissociated protein, it is reasonable to assume that the hysteresis they observed near pH 7 was that which accompanies TMVP association/dissociation (Scheele & Schuster, 1975; also section D2 below). Neither Scheele & Lauffer (1967) nor Butler & Durham (1972) observed hysteresis in their titrations of TMV. In the case of Y-TAMV, titration hysteresis was observed in the present study only if dissociated protein was present in the preparation (see section C(a) below). Hysteresis would thus appear to be a feature of TMV titration curves only if the preparations titrated contained significant quantities of dissociated virus protein.

(ii) Titration behaviour of TMV vulgare

The total of 5.0 protons titrating per subunit between pH 4.5 and pH 8.5 (curve a, Fig. 6A) agreed reasonably well with the figure of 4.6 (at $4^{o}C$) found by Scheele & Lauffer (1967), and the figure of 4.9 found by Butler & Durham (1972). Oehlen (1967) reported that at an ionic strength of I=0.02 only 3.0 protons titrated per subunit between pH 4.5 and pH 8.0. At I=0.1, her results imply that 4.5 protons per subunit titrated over that pH range (Paulsen, 1972). The shape of the titration curve of TMV (curve a, Fig. 6A) was virtually indistinguishable from those obtained by Scheele & Lauffer (1967) and Butler & Durham (1972).

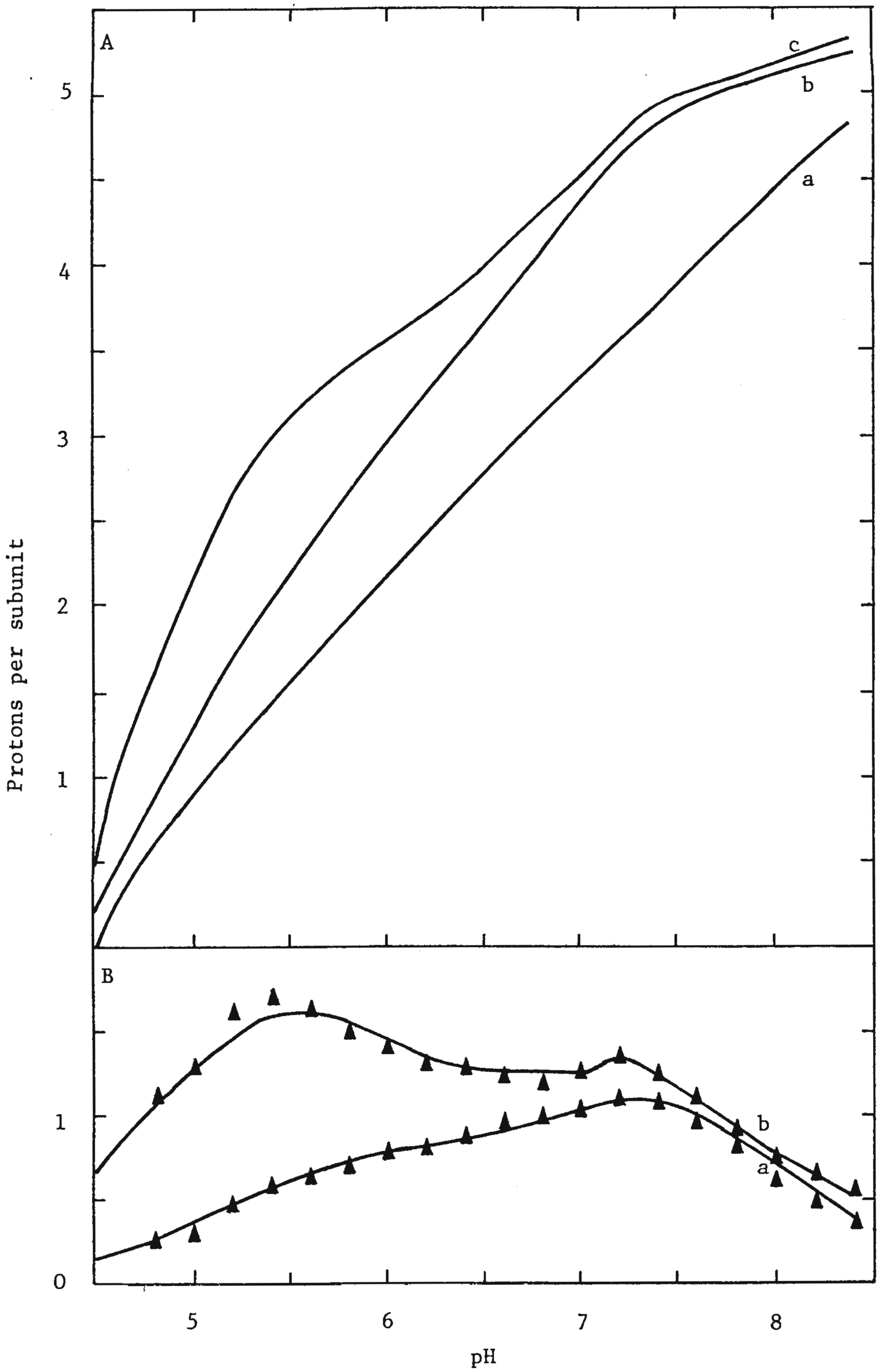


Figure 6. Titration curves of TMV type strain in the presence of calcium.

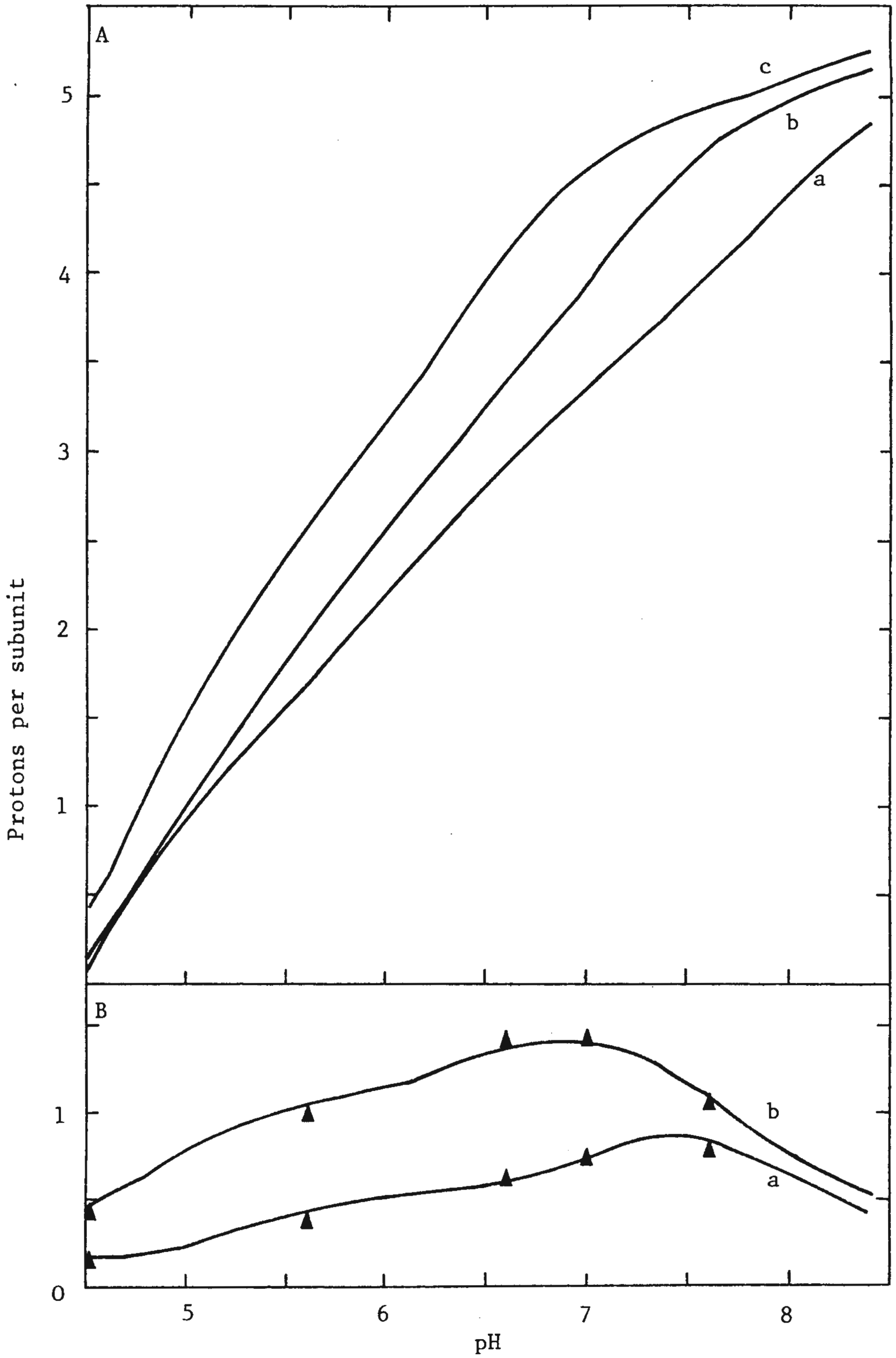


Figure 7. A. Titration curves of TMV type strain in the presence of magnesium. TMV was titrated in the absence of multivalent cations (a), and in the presence of about 1.5 mM Mg^{2+} (b), and about 27 mM Mg^{2+} (c). The curves were positioned vertically using displacement values obtained by Mg^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from TMV by 1.5 mM Mg^{2+} (a) and by 27 mM Mg^{2+} (b) as a function of pH. The smooth curves were derived from the data in Fig. 7(A), and were accurately positioned vertically by means of displacement values obtained by addition of Mg^{2+} to aliquots of TMV (triangles).

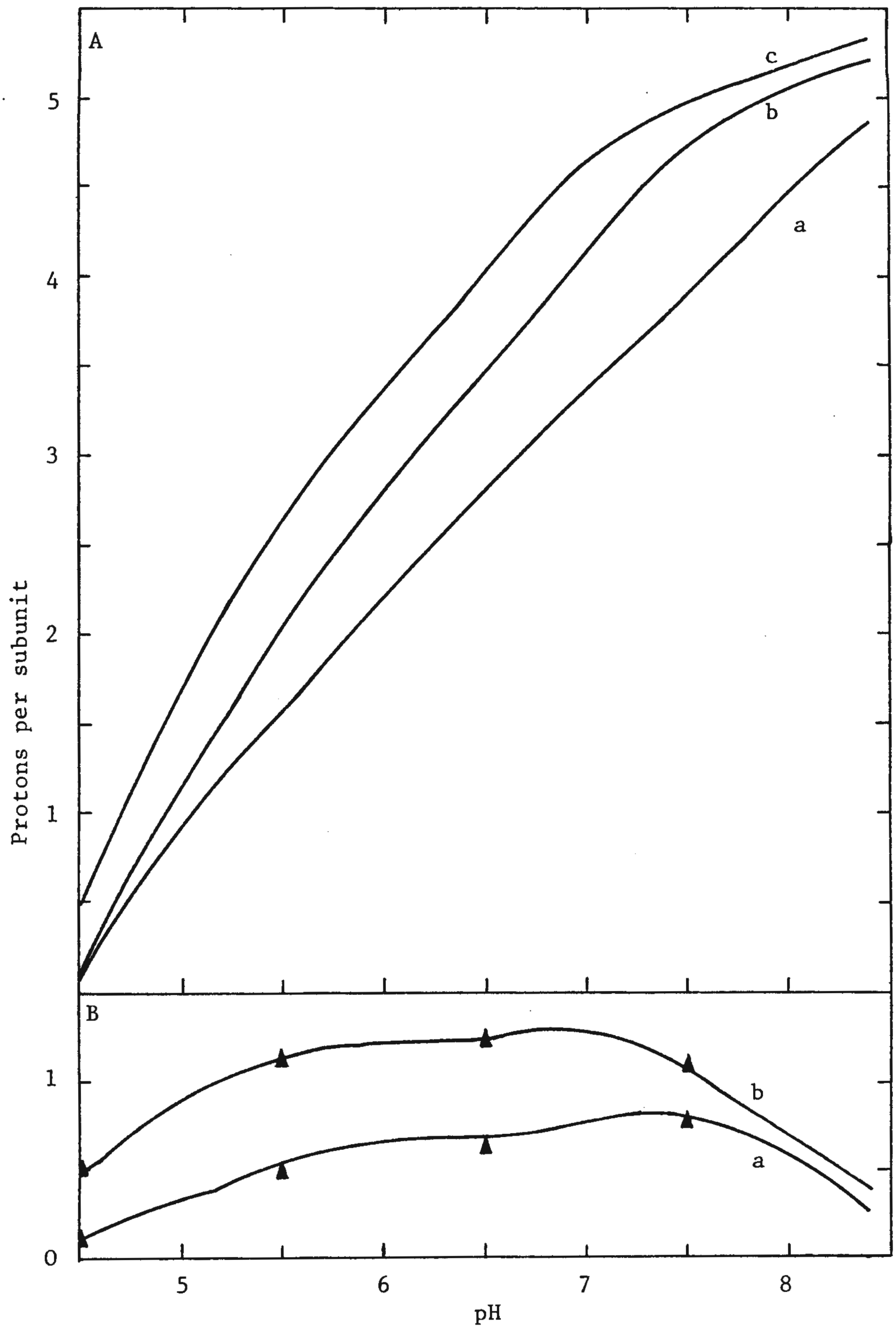


Figure 8. A. Titration curves of TMV type strain in the presence of strontium. TMV was titrated in the absence of multivalent cations (a), and in the presence of about 1.5 mM Sr^{2+} (b), and about 27 mM Sr^{2+} (c). The curves were positioned vertically using displacement values obtained by Sr^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from TMV by 1.5 mM Sr^{2+} (a) and by 27 mM Sr^{2+} (b) as a function of pH. The smooth curves were derived from the data in Fig. 8(A), and were accurately positioned by means of displacement values obtained by addition of Sr^{2+} to aliquots of TMV (triangles).

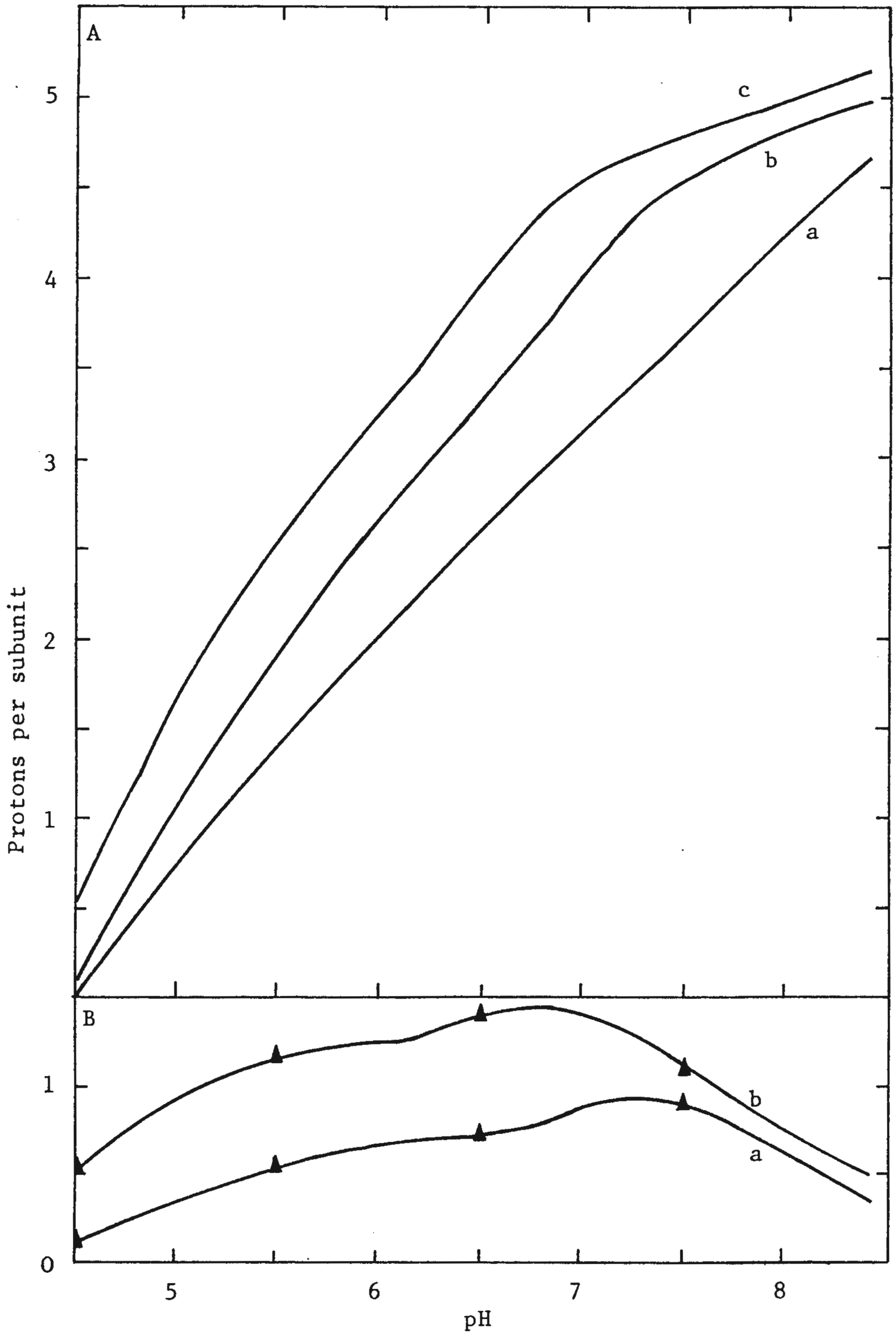


Figure 9. A. Titration curves of TMV type strain in the presence of barium. TMV was titrated in the absence of multivalent cations (a), and in the presence of about 1.5 mM Ba^{2+} (b), and about 27 mM Ba^{2+} (c). The curves were positioned vertically using displacement values obtained by Ba^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from TMV by 1.5 mM Ba^{2+} (a) and 27 mM Ba^{2+} (b) as a function of pH. The smooth curves were accurately positioned vertically by means of displacement values obtained by addition of Ba^{2+} to aliquots of TMV (triangles).

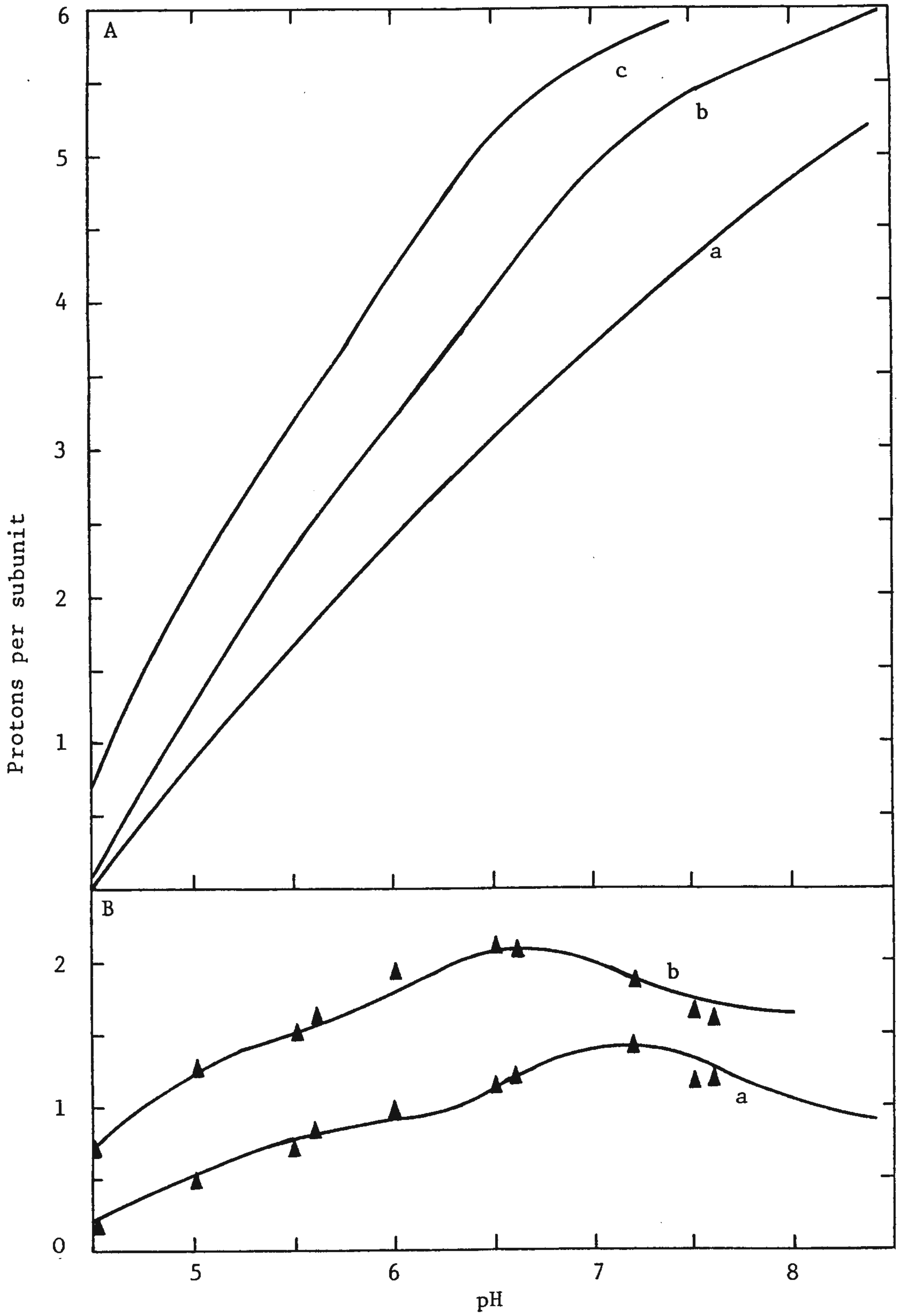


Figure 10. A. Titration curves of TMV type strain in the presence of manganese. TMV was titrated in the absence of multivalent cations (a), and in the presence of about 1.5 mM Mn^{2+} (b), and about 27 mM Mn^{2+} (c). The curves were positioned vertically using displacement values obtained by Mn^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from TMV by 1.5 mM Mn^{2+} (a) and by 27 mM Mn^{2+} (b) as a function of pH. The smooth curves were derived from the data in Fig. 10(A), and were accurately positioned vertically by means of displacement values obtained by addition of Mn^{2+} to aliquots of TMV (triangles).

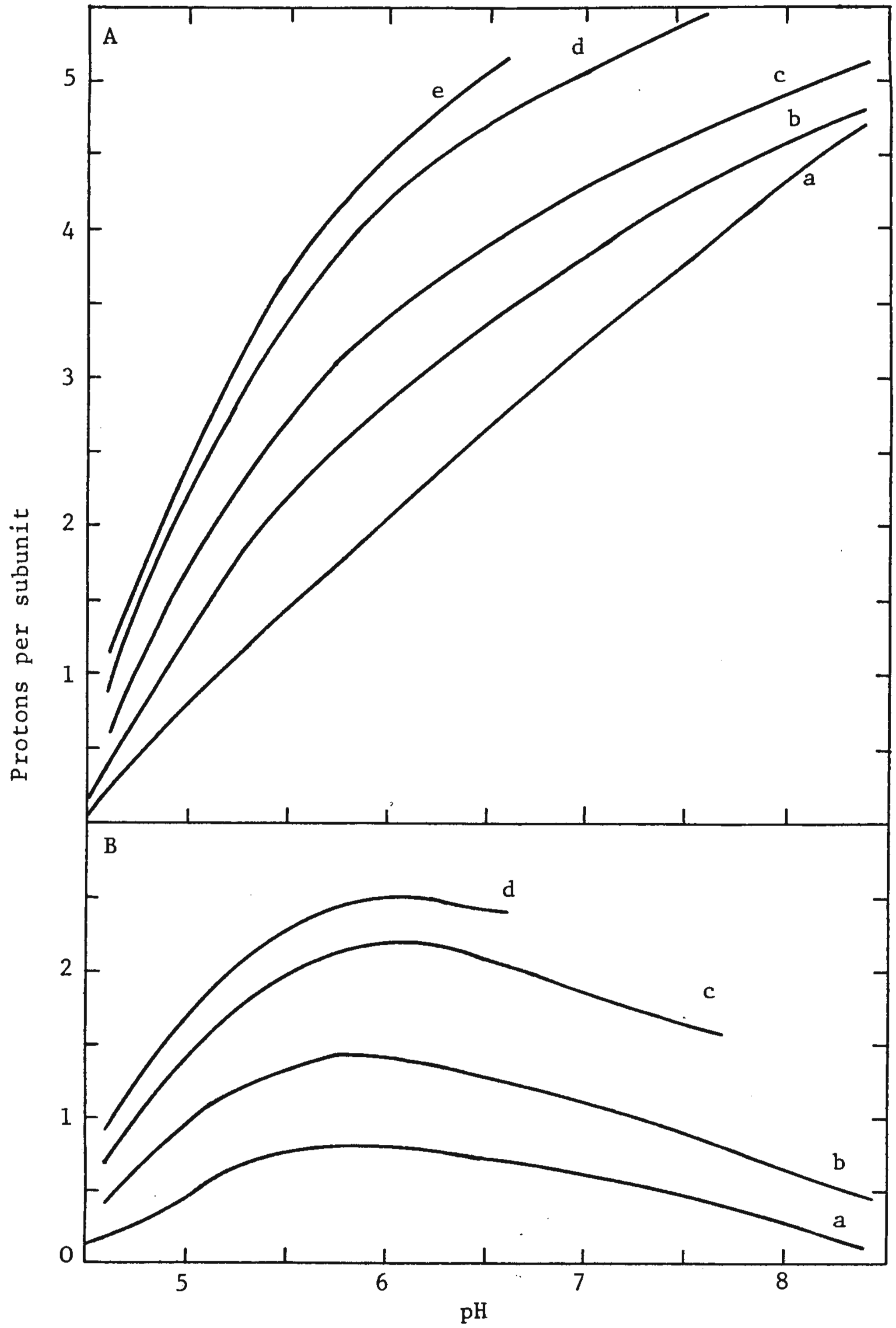


Figure 11. A. Titration curves of TMV type strain in the presence of lead. TMV was titrated alone (a) and in the presence of amounts of $Pb(NO_3)_2$ equivalent to ½, 1, 2 and 3 Pb^{2+} ions per subunit (b, c, d and e, respectively).

B. Differential curves illustrating the number of protons displaced as a function of pH from TMV by amounts of lead equivalent to ½, 1, 2 and 3 (a, b, c and d, respectively) Pb^{2+} ions per subunit. The curves were derived from the data in Fig. 11(A) and were positioned vertically using displacement values obtained by Pb^{2+} addition at pH 4.5.

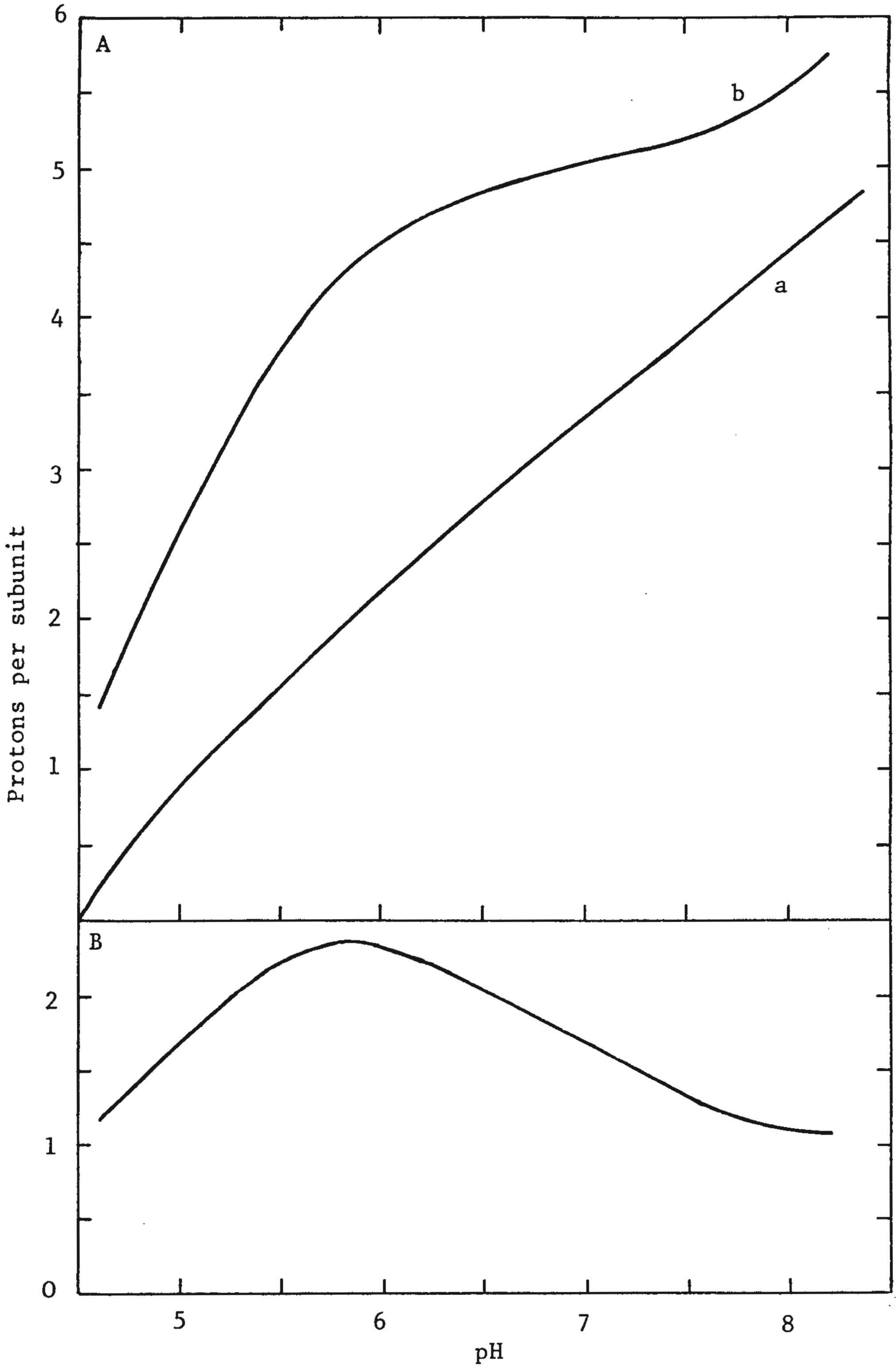


Figure 12. A. Titration curves of TMV type strain in the presence of lanthanum. TMV was titrated alone (a) and in the presence of about 1.5 mM La^{3+} (b).

B. Differential curve illustrating the number of protons displaced from TMV by 1.5 mM La^{2+} as a function of pH. The curve, derived from Fig. 12(A), was positioned vertically by La^{3+} addition at pH 4.5.

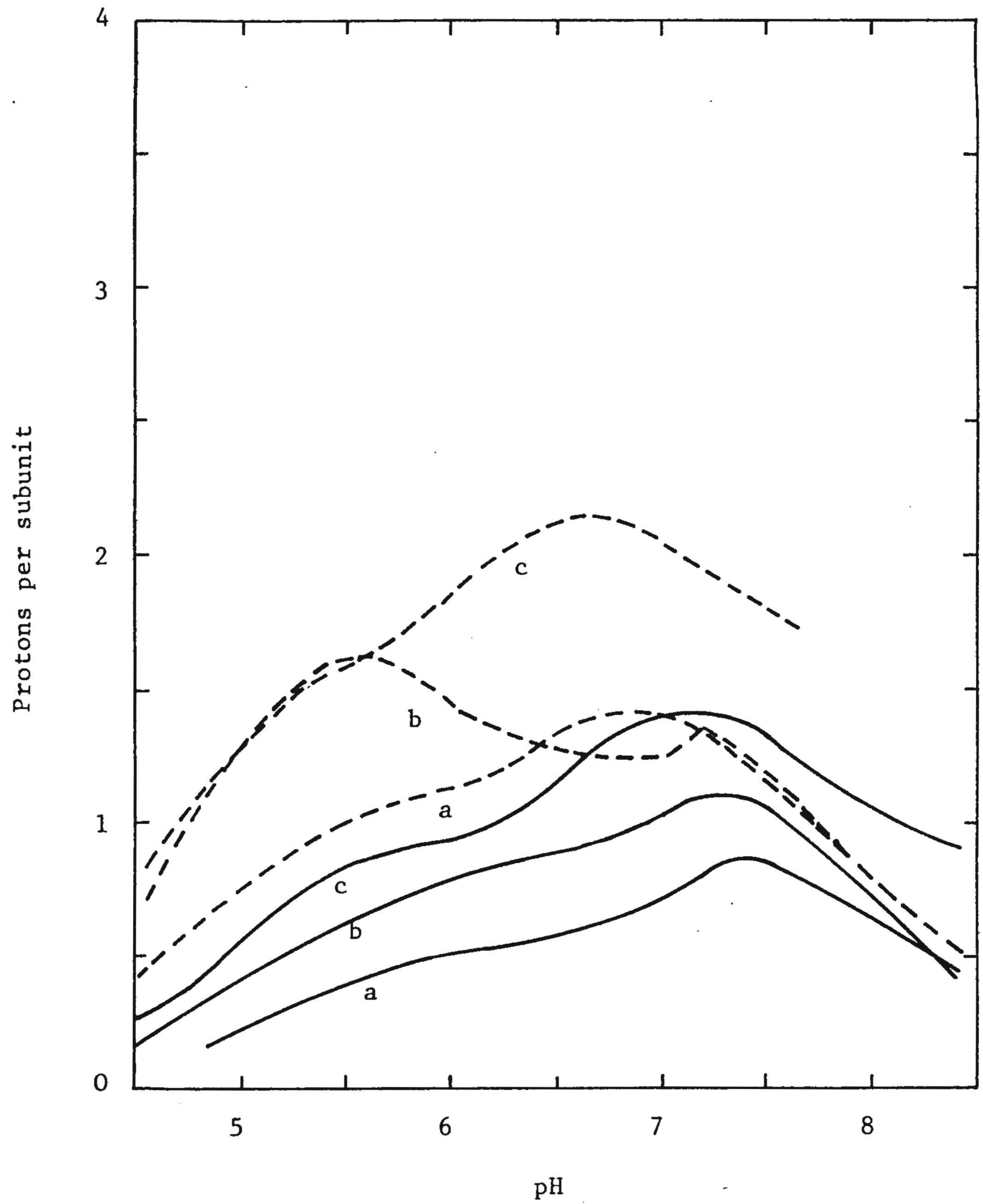


Figure 13. A. The displacement of protons from TMV type strain by divalent cations. The curves, combined for comparison, illustrate the number of protons displaced as a function of pH by 1.5 mM concentrations (solid lines) and 27 mM concentrations (dotted lines) of Mg^{2+} (a), Ca^{2+} (b) and Mn^{2+} (c).

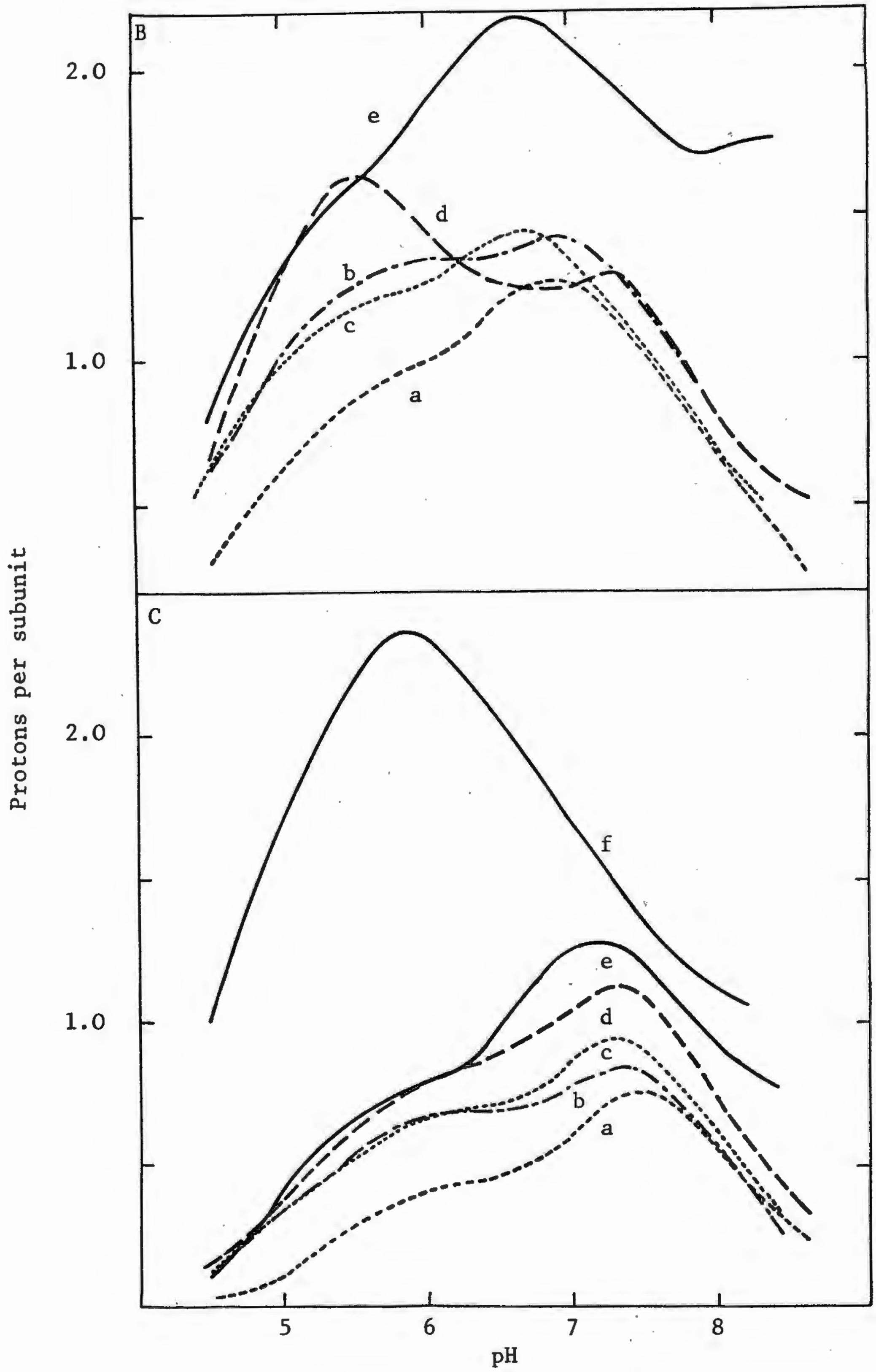


Figure 13B & 13C. The displacement of protons from TMV type strain by multivalent cations. The curves illustrate the number of protons displaced as a function of pH by 1.5 mM concentrations (C) and 27 mM concentrations (B) of strontium (a), magnesium (b), barium (c), calcium (d), manganese (e) and lanthanum (f).

As β- and γ-carboxyl groups have normal pK_H's of 4.08 and 4.50 resp. (Nozaki & Tanford, 1967), it can be calculated (see section C1 of Chapter 2) that at pH 6.0, 97% of the carboxyl groups on the glutamic acid and 99% of those on the aspartic acid residues of a protein should be in the ionised form, assuming normal titration behaviour. Thus, if all six of the functional glutamic acid residues and all eight of the functional aspartic acid residues in TMV vulgare (Wittmann-Liebold & Wittmann, 1967) had titrated normally, not more than 0.26 of the protons titrating above pH 6.0 could have been ascribed to these residues. This figure can be reduced still further if one considers that only eight of these groups in TMV are available for titration (Oehlen, 1967). Similarly, TMV possesses four tyrosine residues, of which only two are ionised (Oehlen, 1967), each with an expected pK_H of 9.6 (Tanford, 1962), although Fairhead et al. (1969) obtained a pK_H of 11.0 for the tyrosine residues in TMV. Assuming normal titration behaviour, a maximum of 0.14 of the protons titrating below pH 8.5 in TMV could thus have been ascribed to the tyrosine residues. The residual basic residues have higher pK_H's and their contribution would have been negligible. In the present study, 2.77 protons titrated per subunit between pH 6.0 and pH 8.5 (curve a, Fig. 6A). Of these, at least 2.37 protons remain unexplained after subtraction of the contribution over this pH range of the carboxyl and tyrosyl groups. Nucleic acids do not normally contain groups titrating near neutrality (Izatt et al., 1971). Thus, TMV must possess more than the previously assumed two anomalously titrating groups per subunit (Caspar, 1963; Butler et al., 1972).

The ability of calcium to displace protons from TMV over a range of four pH units (curve b and c, Fig. 6A; Fig. 6B) suggested that the cation was binding to more than one site per TMVP subunit. This was confirmed by the equilibrium dialysis measurements of calcium binding to TMV (see section B2(a) of this chapter). Calcium binding sites on proteins generally contain at least one carboxylate ligand (Kretsinger & Nelson, 1976). In addition, many precedents exist for carboxyl groups involved in Ca^{2+} binding sites to have raised pK_H values (e.g. Pressman, 1973; Abita _et al._, 1969; Epstein _et al._, 1974). The residues most commonly found to titrate anomalously in proteins are glutamic and aspartic acid, and also tyrosine (Martin, 1964). Glutamic and aspartic acid residues are thus the most likely candidates for the anomalous titration behaviour of TMV (in the light of its amino acid composition, and the results of Oehlen, 1967), and a _prima facie_ case exists for these carboxyl groups being involved in divalent metal binding sites. In fact, Caspar (1963) was the first to suggest that the lead atoms found to bind to TMV were complexed to carboxyl groups with raised pK_H values.

(iii) _Theory of competitive binding_

For each class of sites on a protein, one obtains the competing equilibria:

$$\text{site} + H^+ \rightleftharpoons \text{site-}H^+ \qquad (1)$$

$$\text{and} \quad \text{site} + M^{2+} \rightleftharpoons \text{site-}M^{2+} \text{ (M is any cation)} \qquad (2)$$

which have the respective dissociation constants:

$$K_H = \frac{[H^+][\text{site}]}{[\text{site-}H^+]} \qquad (3)$$

$$\text{and} \quad K_M = \frac{[M^{2+}][\text{site}]}{[\text{site-}M^{2+}]} \qquad (4)$$

For the former, when 50% of the sites are protonated, then:

$$K_H = [H^+]$$

$$\therefore pK_H = pH$$

Similarly, when 50% of the sites are occupied by cation (assuming no protonation of the sites):

$$K_M = [M^{2+}]$$

$$\therefore pK_M = pM$$

Addition of the competing cation to a protein solution results in binding of the metal to the site. The equilibrium position of reaction (1) thus shifts to the left, reducing the degree of protonation of the site. This reduction in protonation can be evaluated as follows:

$$[\text{total sites}] = [\text{site}] + [\text{site-H}^+] + [\text{site-M}^{2+}] \quad (5)$$

but, $[\text{total sites}] = 2 \times [\text{site-H}^+]'$ at the apparent pK_H

$$\therefore [\text{site-H}^+]' = [\text{site}]' + [\text{site-M}^{2+}]' \quad (6)$$

(The primed terms have special and not general values)

From (3) and (4): $$\frac{K_H[M^{2+}]}{K_M[H^+]} = \frac{[\text{site-M}^{2+}]}{[\text{site-H}^+]}$$

Substitute equation (6):

$$\frac{K_H[M^{2+}]}{K_M[H^+]} = \frac{[\text{site-H}^+]' - [\text{site}]'}{[\text{site-H}^+]'}$$

$$= 1 - \frac{K_H}{[H^+]'}, \quad \text{(from equation (3))}$$

$$\therefore \frac{K_H[M^{2+}]}{K_M} = [H^+]' - K_H$$

$$\therefore [H^+]' = K_H \left(1 + \frac{[M^{2+}]}{K_M}\right)$$

= the $[H^+]$ at which 50% of the sites are protonated in the presence of cation M.

$$\therefore \quad pH' = pK_H - \log_{10} \left(1 + \frac{[M^{2+}]}{K_M}\right) \qquad (7)$$

But, if $[M^{2+}] >> K_M$ (i.e. tight metal binding or high metal concentration)

$$\text{then } pH' \simeq pK_H + pM - pK_M$$

$$\therefore \quad pK_H - pH' \simeq pK_M - pM$$

Thus, the addition of metal ions reduces the pH at which 50% of the sites are protonated by approximately (pK_M-pM) units.

From equations (3) and (4) above, we have

$$K_H = \frac{[H^+]\,[\text{site}]}{[\text{site-}H^+]} = \frac{1}{k_H}$$

$$\text{and} \quad K_M = \frac{[M^{2+}]\,[\text{site}]}{[\text{site-}M^{2+}]} = \frac{1}{k_M}$$

$$\therefore \quad [\text{site-}H^+] = k_H\,[H^+]\,[\text{site}] \qquad (8)$$

$$\text{and} \quad [\text{site-}M^{2+}] = k_M\,[M^{2+}]\,[\text{site}] \qquad (9)$$

From equations (5), (8) and (9):

$$[\text{total sites}] = k_H\,[\text{site}]\,[H^+] + k_M\,[\text{site}][M^{2+}] + [\text{site}]$$

$$= [\text{site}]\,(1 + k_H\,[H^+] + k_M\,[M^{2+}])$$

$$\therefore \quad \frac{[\text{site-}H^+]}{[\text{total sites}]} = \frac{k_H\,[\text{site}]\,[H^+]}{[\text{site}](1 + k_H[H^+] + k_M[M^{2+}])}$$

$$= \frac{k_H\,[H^+]}{1 + k_H\,[H^+] + k_M\,[M^{2+}]}$$

But, before addition of M^{2+}:

$$\frac{[\text{site-H}^+]}{[\text{total sites}]} = \frac{k_H\,[\text{site}][H^+]}{[\text{site}]\ (1 + k_H\,[H^+])}$$

$$= \frac{k_H\,[H^+]}{1 + k_H[H^+]}$$

Therefore, upon addition of M^{2+}:

$$[H^+]_{\text{displaced}} = [\text{protein}] \times \sum_{\substack{\text{all}\\ \text{sites}}} \left(\frac{k_H\ [H^+]}{1 + k_H\,[H^+]} - \frac{k_H\,[H^+]}{1 + k_H[H^+] + k_M\,[M^{2+}]}\right)$$

$$= [\text{protein}] \times [H^+] \times \sum_{\substack{\text{all}\\ \text{sites}}} \left(\frac{k_H}{1 + k_H[H^+]} - \frac{k_H}{1 + k_H\,[H^+] + k_M\,[M^{2+}]}\right) \qquad (10)$$

Similarly:

$$\frac{[\text{site-M}^{2+}]}{[\text{total sites}]} = \frac{k_M\,[M^{2+}]}{1 + k_H\,[H^+] + k_M\,[M^{2+}]}$$

$$\therefore\ [M^{2+}]_{\text{bound}} = [\text{protein}] \times [M^{2+}] \times \sum_{\substack{\text{all}\\ \text{sites}}} \left(\frac{k_M}{1 + k_H\,[H^+] + k_M\,[M^{2+}]}\right) \qquad (11)$$

From equation (10), a bell-shaped curve is obtained if one plots as a function of ($pH-pK_H$) the displacement by a metal of protons from a protein containing a single binding site; the shape of the curve varies depending on the value of (pK_M-pM) (Fig. 14). If the protein contains more than one binding site for the metal, proton displacement curves representing the sum of several of the curves in Fig. 14 would be obtained, as in Fig. 13A.

(iv) Results derived from the theory, and their implications

An iterative computer program based on equation (10) above was written by Dr Anthony Durham, assuming that TMV contains three calcium binding sites per TMV molecule. The pK_H and pK_{Ca} values for each site were varied to obtain a least-squares best fit between theoretical values of protons displaced and the values obtained experimentally in 50 mM KCl. The calculated proton displacement curves are shown in Fig. 15 and are based on pK_H figures of 8.33, 7.54 and 5.91 and corresponding pK_{Ca} figures of 5.23, 1.76, and 3.21, for the three sites respectively (see Durham & Hendry, 1977).

From the above pK values and the equations (10) and (11), it can, for example, be calculated that on altering the pH of a solution of TMV in 50 mM KCl at pCa 3 from pH 8.0 to pH 5.0, 1.32 Ca^{2+} ions will be lost and 2.29 protons will be gained per protein subunit. The figure for Ca^{2+} ions lost agrees well with that of 1.31 obtained from the equilibrium dialysis data (Fig. 4). Further, it is of note that the charge alteration between pH 8 and pH 5 due to loss of Ca^{2+} ions is roughly counterbalanced by that due to gain of protons. This feature probably accounts for the relatively constant electrophoretic mobility of TMV over the range pH 5 to pH 8 (Kramer, 1957); due to the compensating binding or dissociation of Ca^{2+} ions over this pH range, the total charge of TMV at pCa values between 2 and 4 is largely unaffected by pH change.

Similarly, it can be calculated that on changing the pCa of a TMV solution (at pH 7.0 in 50 mM KCl) from pCa 3 to pCa 7, each subunit would gain 0.87 protons and lose 1.46 Ca^{2+} ions. The corresponding figure from equilibrium dialysis, done at I=0.1, was 1.04 Ca^{2+} ions (Fig. 4). Consequently, on going from an extracellular to an intracellular environment, a TMV particle

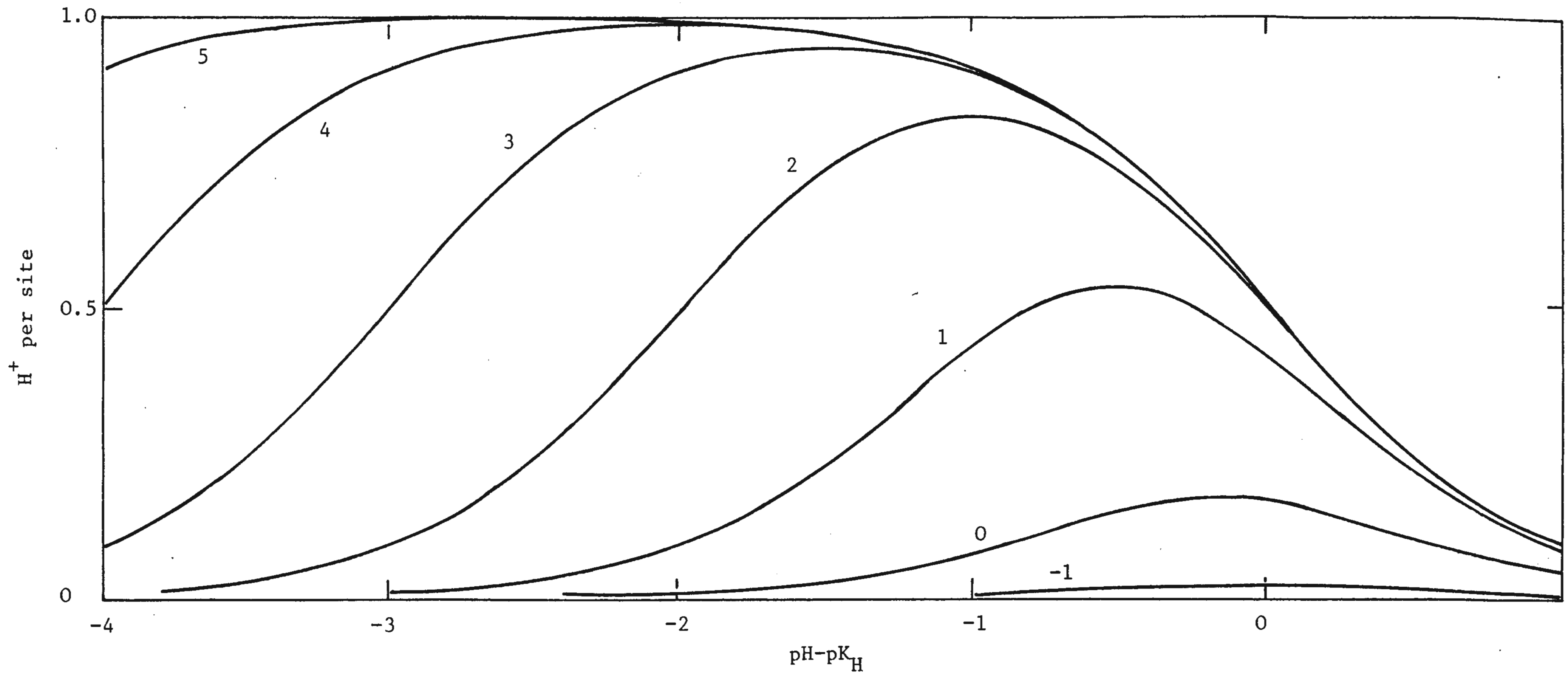


Figure 14. Theoretical curves indicating the displacement of protons from a site by a cation M, as a function of hydrogen ion concentration (pH), cation M concentration (pM), affinity of the site for protons (pK_H) and affinity of the site for M (pK_M). For values of ($pH-pK_H$)>-1, the protons displaceable by M decrease due to the normal dissociation of protons from the site. The protons displaceable by M also decrease as the value of ($pH-pK_H$) decreases, due to increasing competition from protons for the site. This competition can be overcome by an increase in the value of (pK_M-pM), i.e. by an increase in the concentration of M or by an increase in the affinity of the site for M. The curves were derived from equation (10) in the text. Numbers next to the curves indicate (pK_M-pM) values.

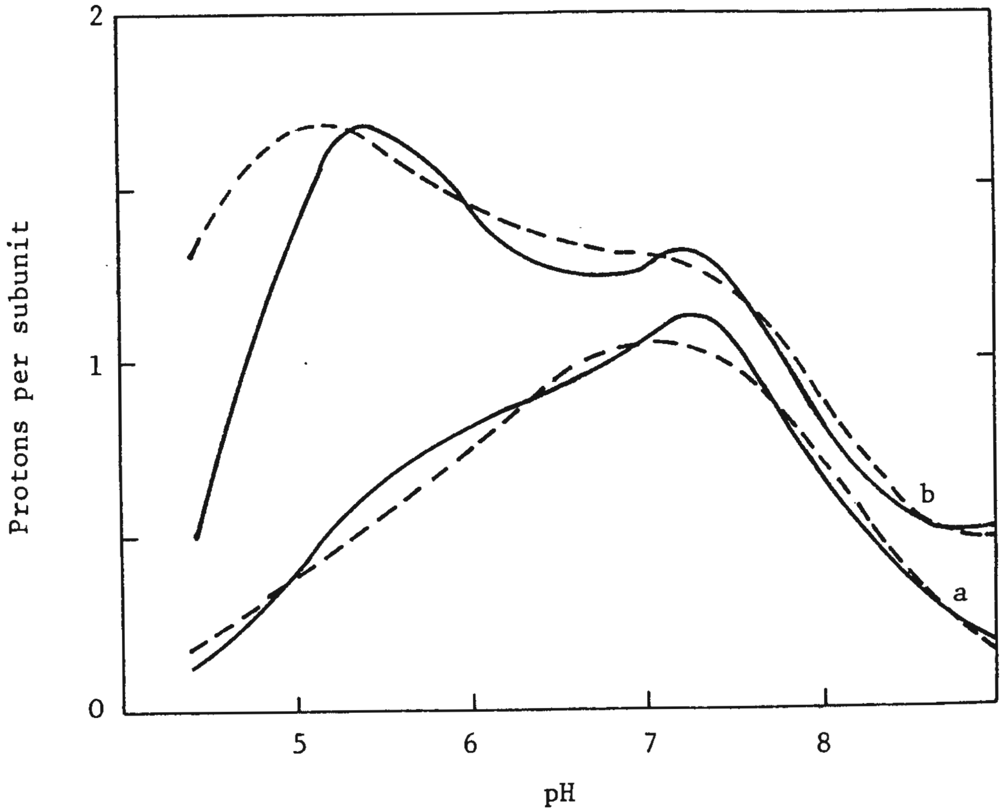


Figure 15. The calculated curves (dotted) compared with the experimental curves (solid lines) for proton displacement from TMV type strain by 1.5 mM Ca^{2+} (a) and by 27 mM Ca^{2+} (b). The calculated curves were obtained by Dr A.C.H. Durham using an iterative computer program based on equation (10) to obtain a least-squares best-fit between theoretical and experimentally-determined values.

would undergo a considerable change in charge. At pH 9.0, the corresponding figures would be 0.14 protons gained and 1.58 Ca^{2+} ions lost, resulting in an increase of more than one negative charge per protein subunit. This effect probably explains the increased TMV degradation at low Ca^{2+} levels observed at pH 9 by Powell (1975), as well as the protective effect of divalent cations at 10^{-3} M concentrations. Powell (1975) in fact stated that charge as well as ionic strength was involved in this protection, as the increased protective effect of divalent cations relative to monovalent cations was more than the three-fold increase that would have been predicted from ionic strength considerations alone.

(v) Affinity of TMV vulgare for other multivalent cations

Applying the same procedure used for the Ca^{2+} titration results to those obtained with Mg^{2+}, Dr A. Durham obtained pK_H values of 8.07, 7.43 and 5.44 and pK_{Mg} values of 4.36, 2.05, and 1.94 respectively (see Durham & Hendry, 1977). These results are based on less data than the Ca^{2+} results, and are less reliable, but they indicate that, except for the pK_H 7.5 site, the TMV sites have a roughly 10-fold stronger affinity for Ca^{2+} than for Mg^{2+}. The binding behaviour of strontium and barium to TMV is similar to that of magnesium (see Figs 7, 8 & 9), strontium being the weakest binder of the three. A feature common to these three cations, and to calcium, is the far higher affinity for these cations of the pK_H 8.3 site on TMV than of the pK_H 5.8 site, as indicated by the greater proton displacement by low concentrations of each cation at pH 7.5 than at pH 5.5 (Fig. 13C). Also, the pK_H 5.8 site has a higher affinity for Ca^{2+} than for Ba^{2+}, Sr^{2+} or Mg^{2+} (Fig. 13B). These facts were subsequently borne out, in the case of Ca^{2+} and Mg^{2+}, by the computer results.

Tobacco mosaic virus had a significantly stronger affinity for manganese than for the previously mentioned cations (Fig. 10). This was particularly evident from the relative proton displacements by 27 mM concentrations of these cations (Fig. 13B). Owing to the similarity below pH 6.5 between the Mn^{2+} and the Ca^{2+} displacement curves at 1.5 mM cation concentrations (Fig. 13C), the high affinity for Mn^{2+} was apparently a function of predominantly the pK_H 8.3 site on TMV. It is noteworthy that the cation affinities for TMV are $Mn^{2+} > Ca^{2+} > Mg^{2+}$, which is the same order reported by Kassanis et al. (1975) for cation-mediated enhancement of protoplast infection by TMV in the presence of chelating agents. They suggested that cations were required by the protoplast membranes, although it is feasible that virus attachment to protoplasts could be a function of the charge on, and therefore the number and type of cations bound to, the TMV particles.

Lead ions displaced protons very strongly from TMV. Curves (b), (c) and (d) in Fig. 11B indicate that each Pb^{2+} ion displaced 1.4 protons at pH 6.0; some Pb^{2+} ions must thus have displaced two protons. The pK_H 5.8 site appeared to have the highest affinity for Pb^{2+} ions, followed by the pK_H 7.5 site and then the pK_H 8.3 site (Fig. 11B). The two highest affinity sites were probably responsible for the lead binding observed at 2.5 nm and 8.4 nm radius by Caspar (1956), although some workers have subsequently reported difficulty in demonstrating lead binding to TMV at the latter site (Durham & Butler, 1975). The aspartic acid residues at positions 115 and 116 in the TMVP polypeptide are thought to contribute to the site at 2.5 nm radius (Butler & Durham, 1972; Holmes et al., 1975).

The trivalent cation lanthanum, which is a recognised probe for sites that bind calcium (Kretsinger & Nelson, 1976), at 1.5 mM concentration mimicked the high proton displacement at pH 5.5 of 27 mM Ca^{2+} (Fig. 12). This high proton displacing ability of lanthanum was probably due to its trivalent nature but also to its having a higher affinity for the pK_H 5.9 site relative to the other sites than did calcium. This feature could make La^{3+} a useful probe for the elucidation of particularly the pK_H 5.9 calcium binding site on TMV.

Based on proton displacing ability, the affinity of divalent cations for TMV is Mn>Ca>Ba>Mg>Sr. Williams (1971b) showed that, although with weak acid anions such as carboxylates the stabilities usually found were Mg>Ca>Sr>Ba, with complex carboxylates such as multidentate ligands any order between that given above and the reverse (Ba>Sr>Ca>Mg; the lyotropic series, found with strong acid anions) was possible. The above series were based on radius-ratio considerations, Mg^{2+} having the smallest ionic radius and Ba^{2+} the largest. The order observed with TMV bears no relation, however, to ionic radii, Mn^{2+} being a small cation with a radius only slightly larger than that of Mg^{2+}. The order thus follows the prediction of Williams (1971b) for a complex carboxylate ligand. The situation regarding TMV is obviously complicated by the fact that what one is observing is the cumulative effect of three binding sites.

The high affinity of TMV for manganese suggests that a nitrogen donor (e.g. on an RNA base) could be involved in the pK_H 8.3 binding site, as manganese, being "softer" than the other cations, has a higher tolerance of nitrogen ligands (Sigel & McCormick, 1970). As mentioned in section B2(c) of Chapter 2, the complexing abilities of metal ions towards nitrogen donors are very different, but are more constant towards oxygen donors. It is interesting in this regard to note that Mg^{2+}, which is more tolerant of nitrogen ligands than is Ca^{2+} (Williams, 1971b), has a slightly higher affinity than Ca^{2+} for the pK_H 7.5 site. The low affinity for TMV vulgare of Mg^{2+} in general is doubtless partly due to the small size of Mg^{2+}. This enables Mg^{2+} ions to polarise water molecules which complex strongly to the cation, thereby increasing the cation's effective radius (Williams, 1970, 1971b).

(vi) General considerations

From the foregoing discussion, it is apparent that both the titration and the metal binding data are best explained by the possession by TMV vulgare of three metal binding sites containing anomalously-titrating groups, probably carboxylates with raised pK_H values. The properties of these sites at I=0.05 can be summarised as:

	pK_H	pK_{Ca}	pK_{Mg}
Site 1	8.3	5.2	4.4
Site 2	7.5	1.5	2.0
Site 3	5.9	3.1	1.9

The pK_{Ca} values indicate that when a TMV particle enters the cytoplasm of a cell, with a pCa of the order of 6-7, virtually 100% dissociation of Ca^{2+} ions from all the sites will occur. If the virus particle had been in contact with water or extracellular fluids, site 1 and possibly site 3 will have contained bound calcium before entry into the cell. Dissociation of these ions from the virion has been shown in the present study to be insufficient to be the sole cause of virus disassembly; however, bound cations have been shown to stabilise TMV (Powell, 1975) and would probably inhibit TMV disassembly in extracellular environments. The dissociation of the calcium ions would, however, produce a considerable alteration in the charge of the TMV particle, amounting to the increase of one negative charge per TMV subunit at pH 7.5. As discussed in section four of this section, this charge effect probably explains the observations of Powell (1975) regarding the ability of divalent cations to counteract the alkaline degradation of TMV: bound divalent cations considerably reduced the negative charge and therefore the mutual repulsion of the protein subunits.

The observation that alkaline degradation of TMV commences above pH 8 (section B1 of this chapter; Brakke & van Pelt, 1969) suggests that the group with a pK_H of 8.3 controls the <u>in vitro</u> stability of the TMV particle. This inference is supported by the observation (see section D2 of this chapter) that this group is absent on TMVP. The pK_H 8.3 site thus appears to involve RNA and protein, and perhaps also a divalent cation stabilising a bond between these two components. Wacker <u>et al</u>. (1963), in fact, concluded that metals were firmly bound to TMV by chelation to the RNA bases.

Evidence supporting this possibility was presented by Stubbs et al. (1977) who reported that most, if not all, of the anomalously titrating groups on TMV originate in a "carboxyl cage" situated in close proximity to the RNA. Site 1 binds calcium with a hundred-fold greater affinity than site 3, and would thus be the last site to lose its bound calcium. This could conceivably be the final in vivo event prior to disassembly, the removal of calcium "unlocking" an RNA-protein bond and increasing the repulsive charge on the individual subunits to a level that forces them apart.

The obvious counter to the argument that this disassembly occurs in the cytoplasm is the fact that TMV particles are known to assemble and pack into crystals in the cytoplasm. Virus particles are thus obviously stable in that milieu, and the postulated disassembly events would have to occur in another part of the cell. Durham (manuscript submitted for publication) has suggested that this could occur at the cell membrane, and has reasoned that the free energy made available to the virion by the dissociation of calcium ions would be sufficient to drive both virus penetration and the disassembly process.

It is unlikely that the low levels of binding observed with barium, strontium and magnesium (all alkaline earth metals like calcium) have any biological significance. Both site 1 and site 3 have a ten-fold lower affinity for Mg^{2+} ions than for Ca^{2+} ions. Particles of TMV infecting a cell are thus more likely to have calcium ions bound to these sites than magnesium ions. To prevent the in vivo attachment of Mg^{2+} ions to these sites from countering the suggested destabilising effects of Ca^{2+} dissociation from the virion, the Mg^{2+} concentration at the intracellular site of uncoating would have to be not more than ten times the Ca^{2+} concentration. The high affinity of TMV for Mn^{2+} ions probably has no biological function under normal circumstances. The efficiency of Mn^{2+} ions in enhancing the infection of protoplasts by TMV (Kassanis _et al._, 1975) can be explained as being due to the ability of Mn^{2+} ions to bind strongly to TMV, thereby affecting the charge and thus the attachment to protoplasts, of the TMV particle.

TMV thus possesses cation-binding sites having an affinity for calcium such that the _in vivo_ dissociation of that metal from the virion could play a role in virus disassembly. Removal of calcium ions is a necessary but not sufficient condition for RNA and protein to actually separate _in vitro_, and other factors are probably involved _in vivo_ as well. The affinity of these sites for other cations is sufficiently low to prevent their binding to them from having any biological role.

The cation-binding characteristics of the U2, Y-TAMV and cowpea strains of TMV will be presented in the following section.

C. Strains of tobacco mosaic virus

The type, U2, Y-TAMV and cowpea strains of TMV have been shown to bind calcium ions (section B2 of this chapter), and the binding of various cations to vulgare has been characterised by hydrogen-ion titration (section B3 of this chapter). This technique was then used to examine the binding of cations to the other three above-mentioned TMV strains.

1. Titration of TMV strains

(a) Titration of Y-TAMV

The acid-base titration curve of divalent cation-free Y-TAMV (curve a, Fig. 16A) was very similar to that of type strain. No hysteresis was detectable between the forward and reverse titration curves. Initially, hysteresis was detected, but was subsequently found to be due to the presence of dissociated protein in the preparations. (See Materials and Methods, section C4). A total of 4.8 protons per subunit titrated between 4.5 and pH 8.5.

Protons were displaced from Y-TAMV between pH 4.5 and pH 8.5 by the addition of calcium (Fig. 16A & 16B), magnesium (Fig. 17A & 17B) and manganese Fig. 18A & 18B). The displacement curves appropriate to each metal were very similar to the corresponding curves for type strain, except that each cation displaced between 0.1 and 0.2 protons per subunit more from Y-TAMV than from type strain. The exception was calcium, which at 27 mM concentration showed a single, broad maximum of proton displacement from Y-TAMV at pH 6.1 (curve b, Fig. 16B) as opposed to the two maxima at pH 5.5 and pH 7.2 in the case of type strain (curve b, Fig. 6B). As was observed with type strain, manganese at 27 mM concentration displaced protons from Y-TAMV more strongly than did either calcium or magnesium.

The differential curves illustrating proton displacement from Y-TAMV by the three cations are combined in Fig. 19 for comparison.

(b) <u>Titration of the U2 strain of TMV</u>

The acid-base titration curve of divalent cation-free U2 strain (curve a, Fig. 20A) differed from that of type strain. The type strain titration curve was approximately linear between pH 4.5 and pH 8.5 (curve a, Fig. 6A) indicating an essentially uniform buffering capacity over that pH range. Between pH 4.8 and pH 6.5, the titration curve of the U2 strain had a shallower slope, indicative of a diminished buffering capacity,i.e. fewer titratable protons, than the corresponding region on the type strain curve.

This is illustrated by the fact that, between pH 4.8 and 6.5, 1.3 protons titrated per U2 protein subunit as compared to the 2.3 protons per protein subunit of type strain. Above pH 6.5 the slopes of the U2 and type strain titration curves were comparable, with about 2.2 protons per subunit titrating between pH 6.5 and pH 8.5 in each case. A total of 3.8 protons per subunit titrated between pH 4.5 and pH 8.5.

The displacement of protons from the U2 strain by calcium (Fig. 20A & 20B) magnesium (Fig. 21A & 21B) and manganese (Fig. 22A & 22B) was greater than the corresponding displacements in the case of type strain. Manganese displaced a maximum of 3.7 protons per subunit at pH 6.4 (curve b, Fig. 22B), calcium a maximum of 3.2 protons per subunit at pH 6.2 (curve b, Fig. 20B), and magnesium a maximum of 3.0 protons per subunit at pH 6.6 (curve b, Fig. 21B). At each given cation concentration, at least one proton per subunit more was displaced from U2 than was displaced from type strain. For each cation, the pH of maximum proton displacement from U2 strain was about 0.3 pH units lower than that from type strain, except that proton displacement from U2 by calcium was maximal at pH 6.3 as opposed to pH 5.5 in the case of type strain.

The differential curves illustrating proton displacement from U2 strain (combined in Fig. 23 for comparison) were markedly similar, and the higher cation concentration in each case resulted in the pH of maximum proton displacement shifting to about 0.4 pH units lower than that of maximum proton displacement by the lower concentration.

(c) Titration of the cowpea strain of TMV

The acid-base titration curve of divalent cation-free cowpea strain (curve a, Fig. 24A) resembled that of U2 strain more closely than that of type strain. In common with the type, U2 and Y-TAMV strains, no hysteresis was detectable. A total of 5.2 protons per subunit titrated between pH 4.5 and pH 8.5. Of these, 1.3 protons per subunit titrated between pH 4.5 and pH 6.0, and 3.9 protons per subunit titrated between pH 6.0 and pH 8.5. These two displacement figures should be compared respectively with the figures of 2.1 and 2.9 protons per subunit titrating in the case of type strain (curve a, Fig. 6A). Above pH 6.0, cowpea strain thus had an increased buffering capacity (i.e. more titratable protons) relative to type strain, while below pH 6.0 cowpea strain had a diminished buffering capacity.

Protons were displaced from cowpea strain between pH 4.5 and pH 8.5 by the addition of calcium (Fig. 24A & 24B) magnesium (Fig. 25A & 25B) and manganese (Fig. 26A & 26B). The proton displacement was higher than that from type strain but not as high as that from U2 strain. Calcium displaced a maximum of 3.2 protons per subunit at pH 6.8 (curve b, Fig. 24B), manganese a maximum of 3.2 protons per subunit at pH 6.7 (curve b, Fig. 26B) and magnesium a maximum of 1.8 protons per subunit at pH 7.1 (curve b, Fig. 25B). A feature of the proton displacement by magnesium was the slow reactivity in the region of pH 5.8. Following the addition of the metal ion, proton release (i.e. a steady drop in pH) occurred for up to 60 minutes. At pH 5.8, proton displacement by calcium and manganese was complete within one or two minutes.

For each cation except calcium, the pH of maximum proton displacement from cowpea strain was similar to that from type strain. The higher cation concentration in each case resulted in the pH of maximum proton displacement shifting to about 0.5 pH units lower than the pH of maximum proton displacement by the lower concentration (Fig. 27).

The characteristics of proton displacement from the four TMV strains by calcium, magnesium and manganese are listed in Table 14. Proton displacements from the four TMV strains by calcium, magnesium and manganese are illustrated for comparison in Figs 28, 29 and 30 for each cation respectively.

TABLE 14

The characteristics of proton displacement from the four TMV strains by calcium, magnesium and manganese

Cation	[Cation] (mM)[a]	Vulgare ΔH_{max}[b]	Vulgare pH[c]	Y-TAMV ΔH_{max}[b]	Y-TAMV pH[c]	U2 ΔH_{max}[b]	U2 pH[c]	Cowpea ΔH_{max}[b]	Cowpea pH[c]
Ca	1.5	1.1	7.3	1.2	7.3	2.6	6.6	2.0	7.3
	27	1.6	5.5	1.8	6.1	3.2	6.2	3.2	6.8
Mg	1.5	0.9	7.4	0.9	7.6	1.9	7.4	1.0	7.5
	27	1.4	6.9	1.6	6.8	2.8	6.6	1.8	7.1
Mn	1.5	1.4	7.2	1.6	7.0	3.0	6.6	2.3	7.2
	27	2.2	6.7	2.4	6.4	3.7	6.4	3.2	6.7

[a]The figure represents the final, total concentration of cation in the titration vessel.

[b]ΔH_{max} is the maximum number of protons per subunit displaced by the cation.

[c]The pH at which the maximum number of protons is displaced.

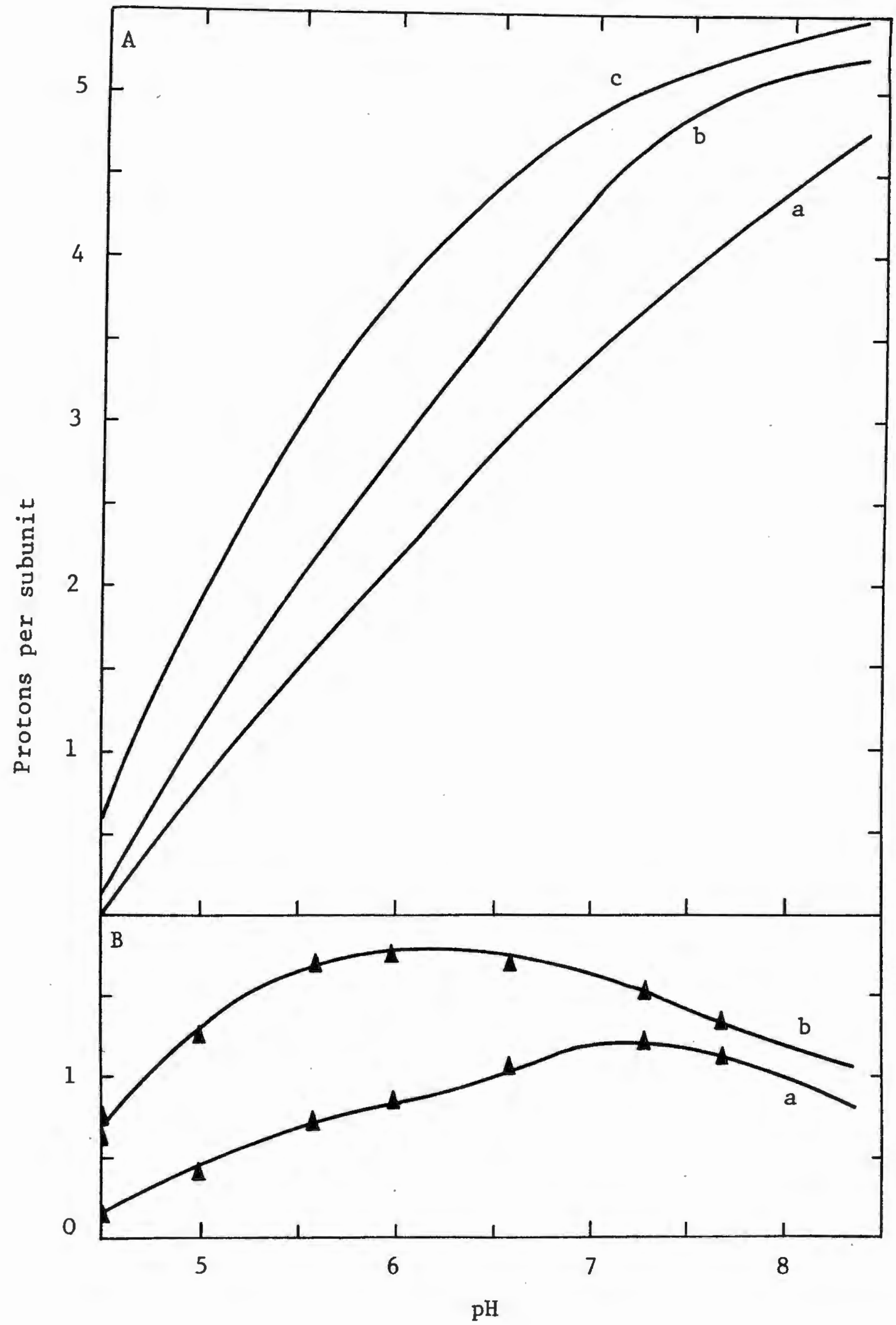
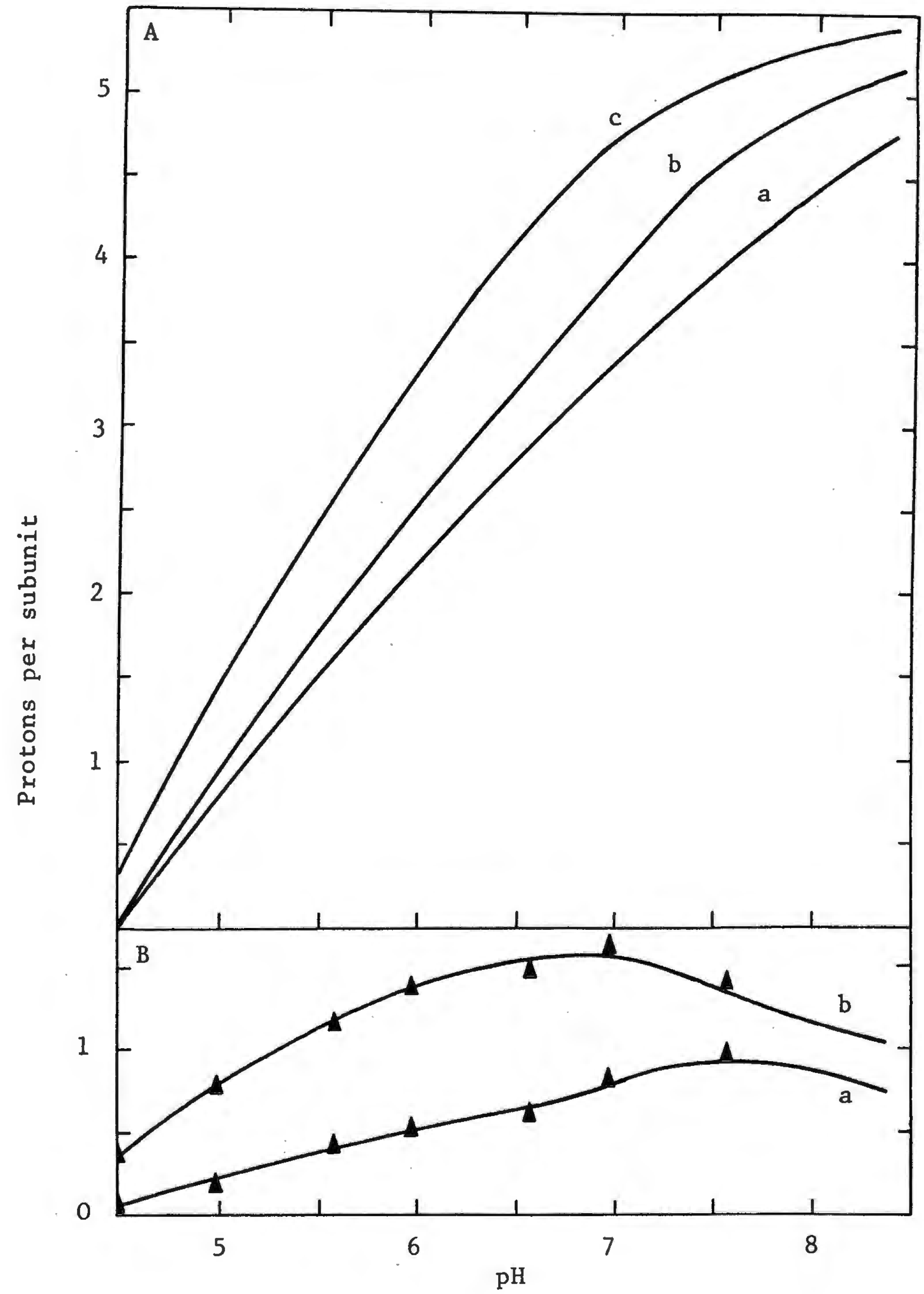


Figure 16. A. Titration curves of Y-TAMV in the presence of calcium. Y-TAMV was titrated alone (a), and in the presence of about 1.5 mM Ca^{2+} (b) and about 27 mM Ca^{2+} (c). The curves were positioned vertically using displacement values obtained by Ca^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from Y-TAMV by 1.5 mM (a) and 27 mM (b) Ca^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 16(A) and were accurately positioned by means of displacement values obtained by addition of Ca^{2+} to aliquots of Y-TAMV (triangles).



<u>Figure 17.</u> A. Titration curves of Y-TAMV in the presence of magnesium. Y-TAMV was titrated alone (a), and in the presence of about 1.5 mM Mg^{2+} (b) and about 27 mM Mg^{2+} (c). The curves were positioned vertically using displacement values obtained by Mg^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from Y-TAMV by 1.5 mM (a) and 27 mM Mg^{2+} (b) as a function of pH. The smooth curves were derived from the data in Fig. 17(A) and were accurately positioned by means of displacement values obtained by addition of Mg^{2+} to aliquots of Y-TAMV (triangles).

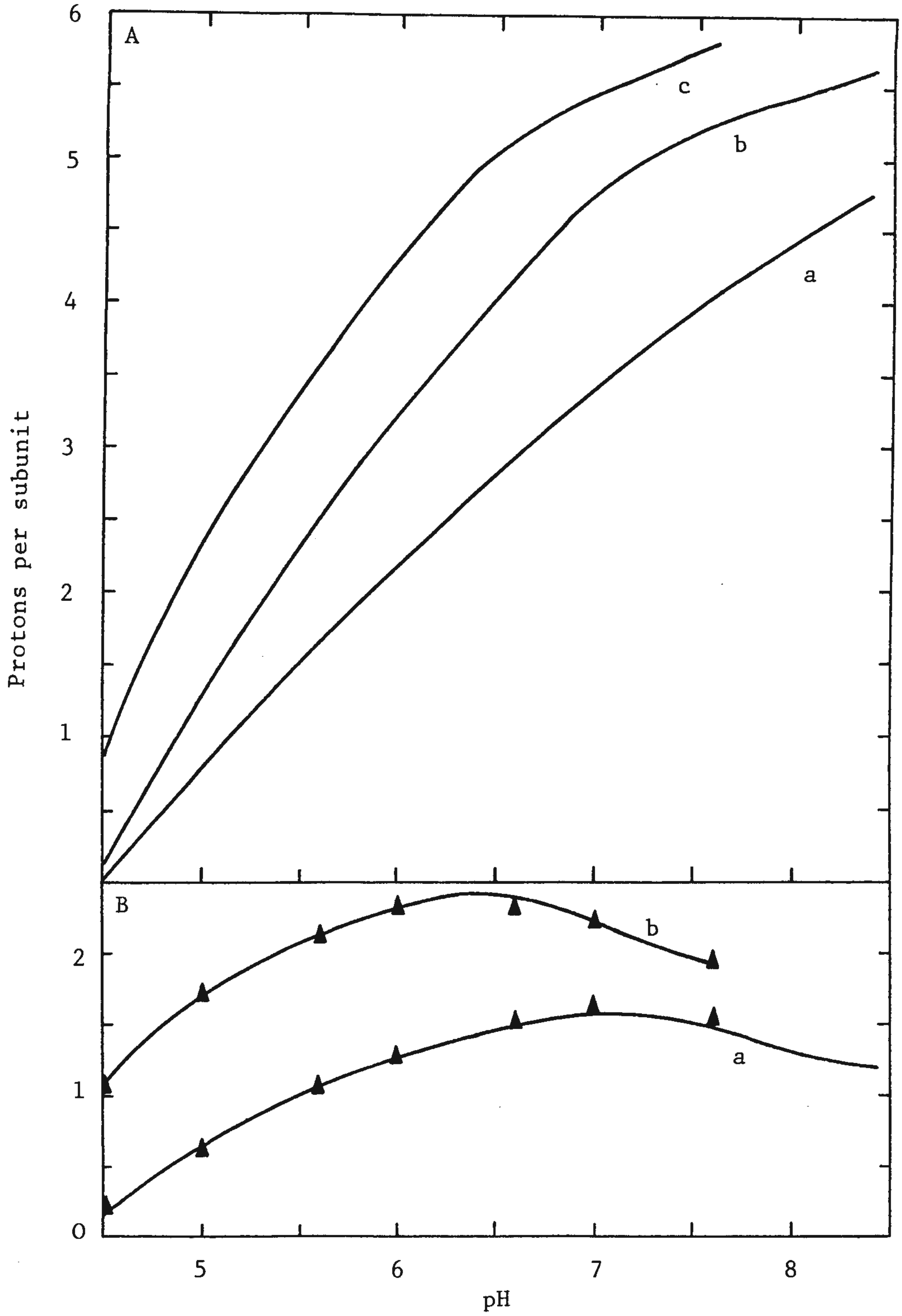


Figure 18. A. Titration curves of Y-TAMV in the presence of manganese. Y-TAMV was titrated alone (a), and in the presence of about 1.5 mM Mn^{2+} (b) and about 27 mM Mn^{2+} (c). The curves were positioned vertically using displacement values obtained by Mn^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from Y-TAMV by 1.5 mM (a) and 27 mM (b) Mn^{2+} as a function of pH. The smooth curves were derived from data in Fig. 18(A) and were accurately positioned by means of displacement values obtained by addition of Mn^{2+} to aliquots of Y-TAMV (triangles).

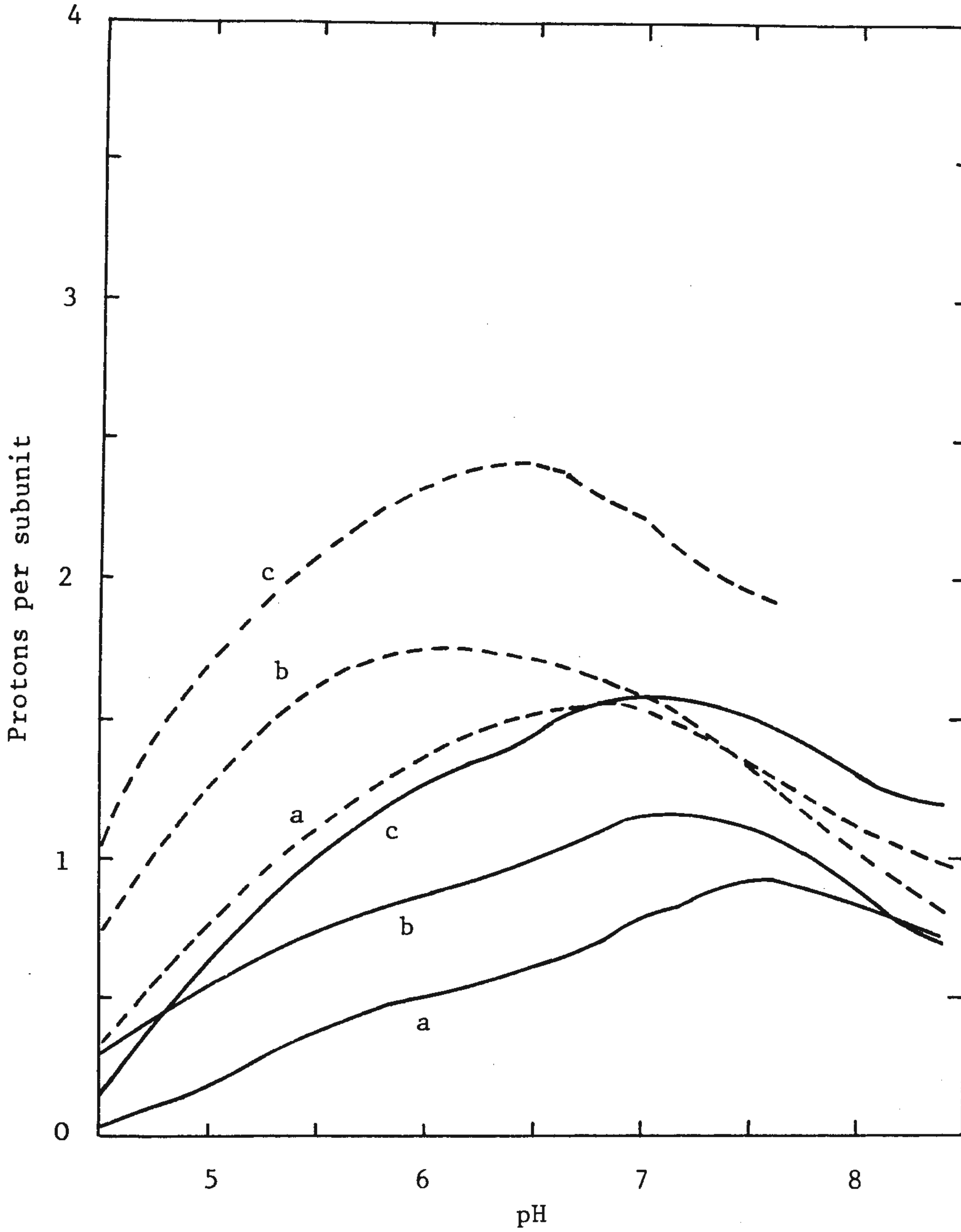


Figure 19. The displacement of protons from Y-TAMV by divalent cations. The differential curves, combined for comparison, illustrate the number of protons displaced as a function of pH from Y-TAMV by 1.5 mM concentrations (solid lines) and 27 mM concentrations (dotted lines) of Mg^{2+} (a), Ca^{2+}(b) and Mn^{2+} (c).

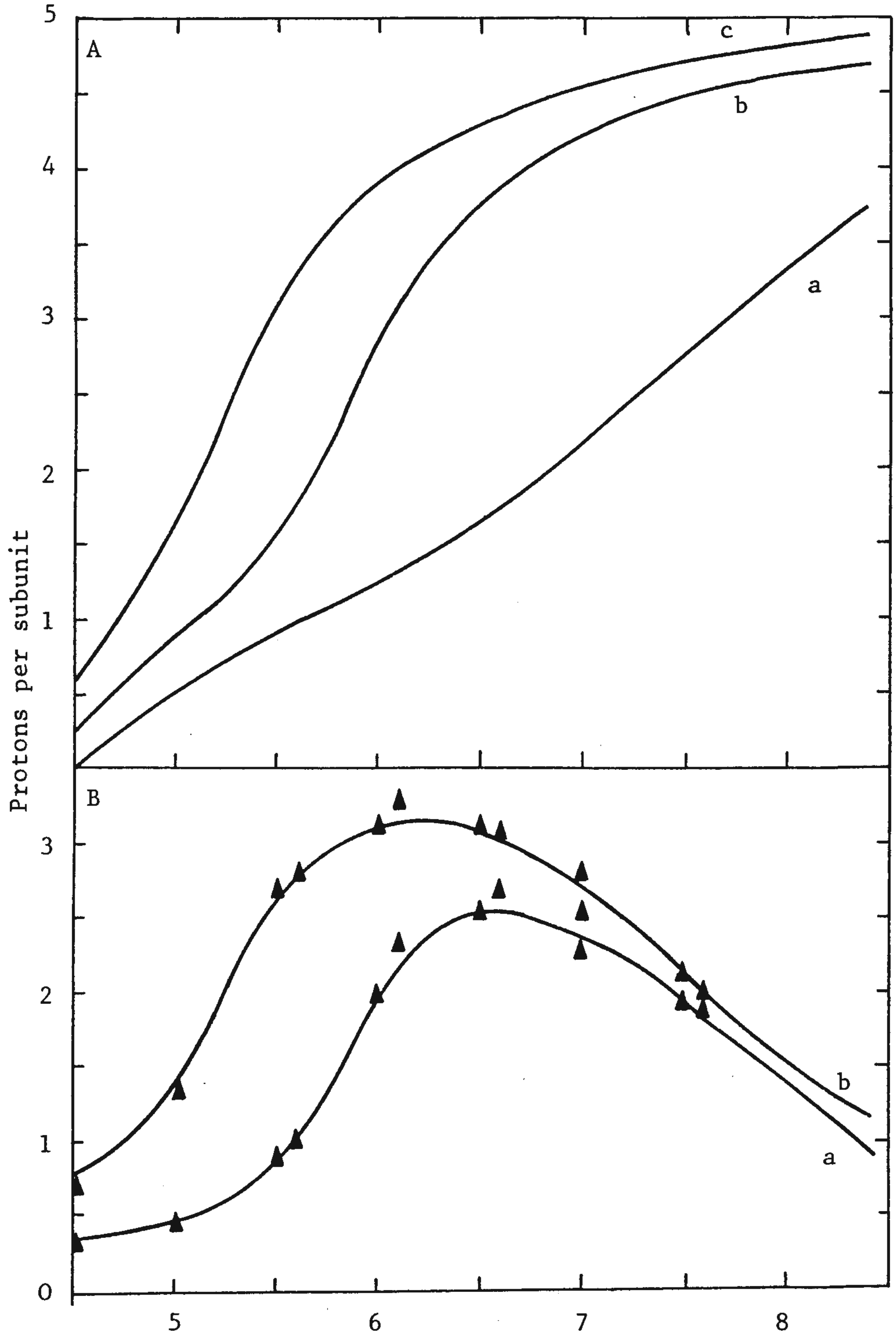


Figure 20. A. Titration curves of the U2 strain of TMV in the presence of calcium. U2 was titrated alone (a), and in the presence of 1.5 mM Ca^{2+} (b) and 27 mM Ca^{2+} (c). The curves were positioned vertically using displacement values obtained by Ca^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from U2 by 1.5 mM (a) and 27 mM (b) Ca^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 20(A) and were accurately positioned by means of displacement values obtained by addition of calcium to aliquots of U2 (triangles).

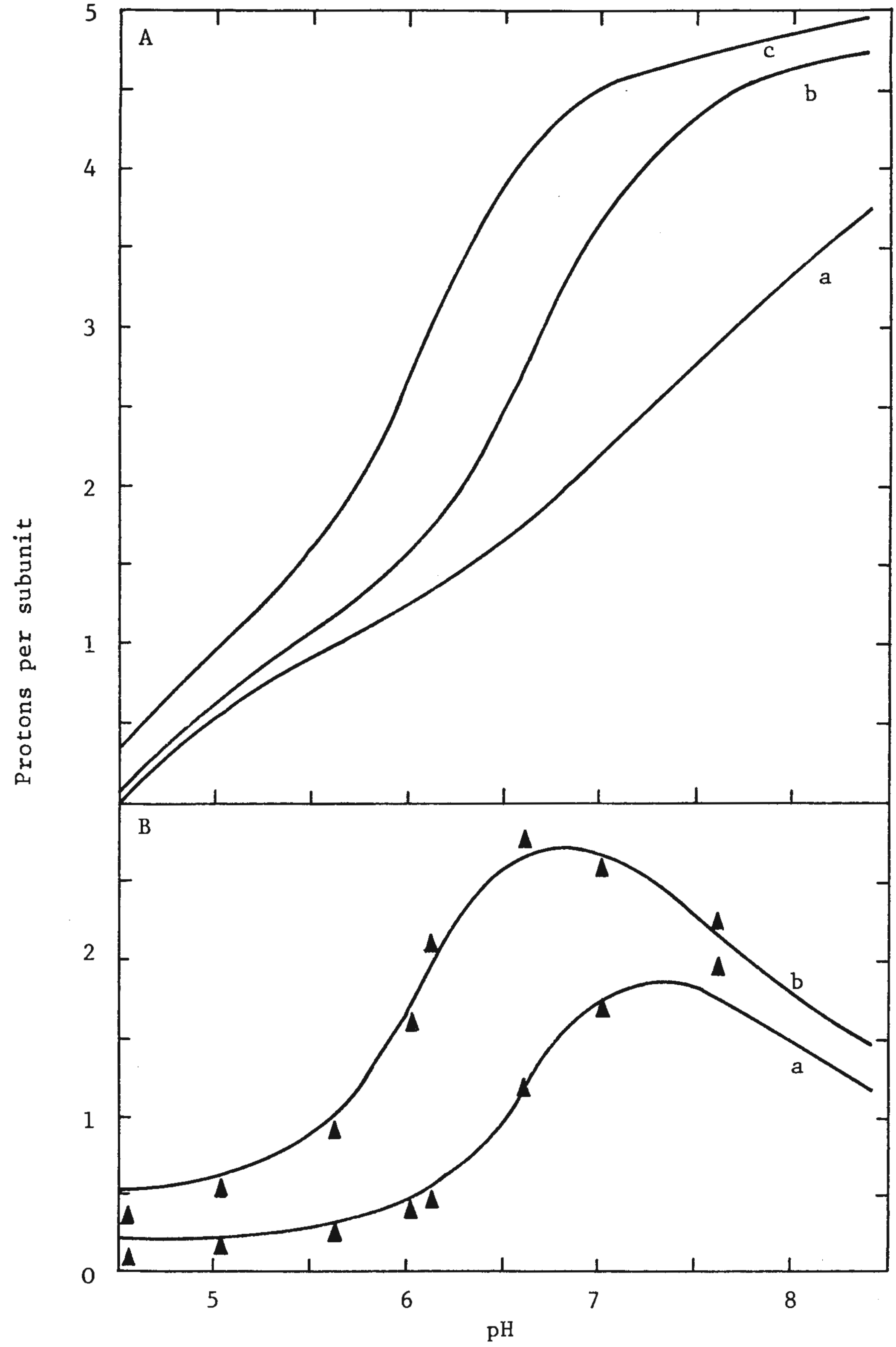


Figure 21. A. Titration curves of the U2 strain of TMV in the presence of magnesium. U2 was titrated alone (a), and in the presence of 1.5 mM Mg^{2+} (b) and 27 mM Mg^{2+} (c). The curves were positioned vertically using displacement values obtained by Mg^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from U2 by 1.5 mM (a) and 27 mM (b) Mg^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 21(A) and were accurately positioned by means of displacement values obtained by addition of magnesium to aliquots of U2 (triangles).

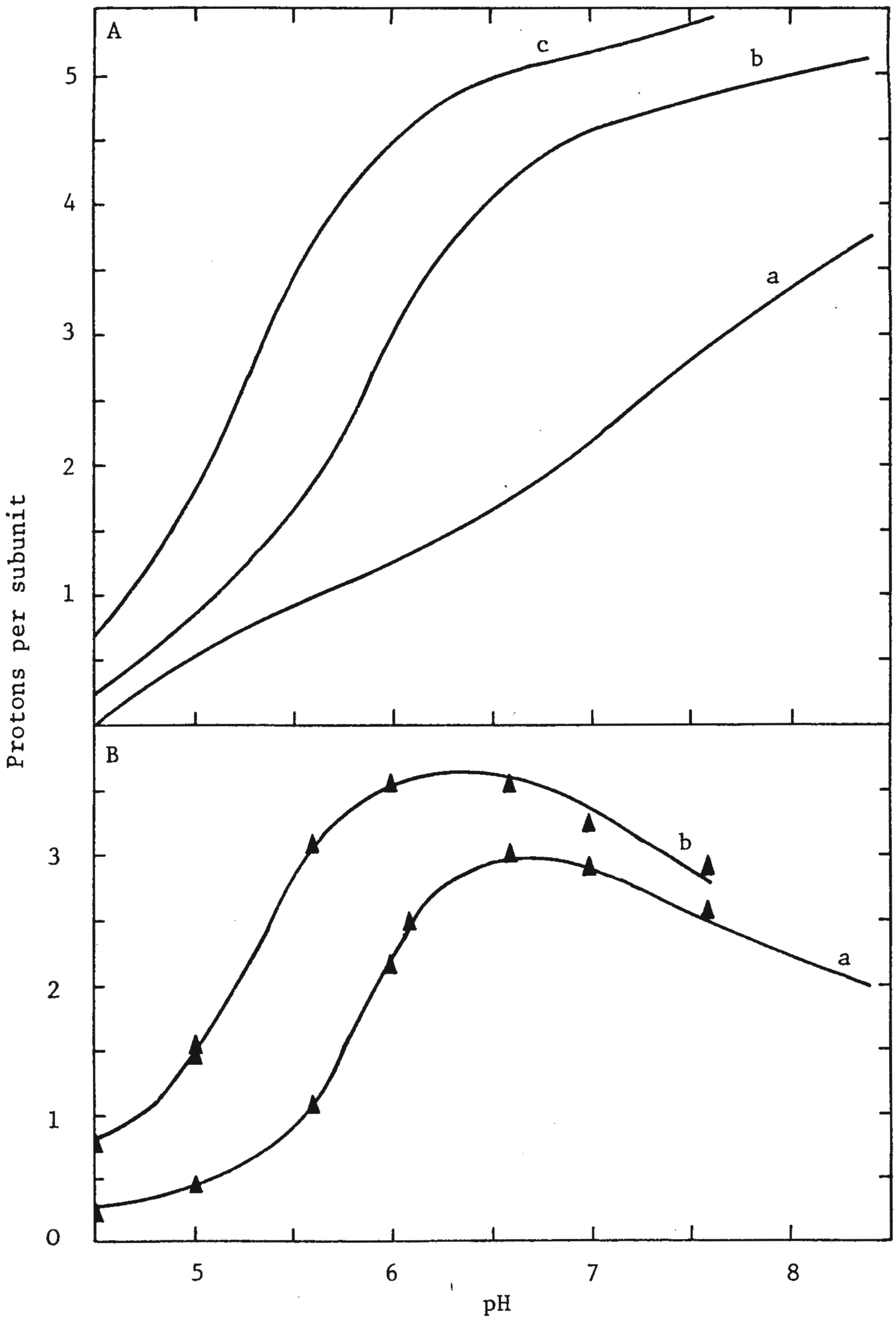


Figure 22. A. Titration curves of the U2 strain of TMV in the presence of manganese. U2 was titrated alone (a), and in the presence of 1.5 mM Mn^{2+} (b) and 27 mM Mn^{2+} (c). The curves were positioned vertically using displacement values obtained by Mn^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from U2 by 1.5 mM (a) and 27 mM (b) Mn^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 22(A) and were accurately positioned by means of displacement values obtained by addition of manganese to aliquots of U2 (triangles).

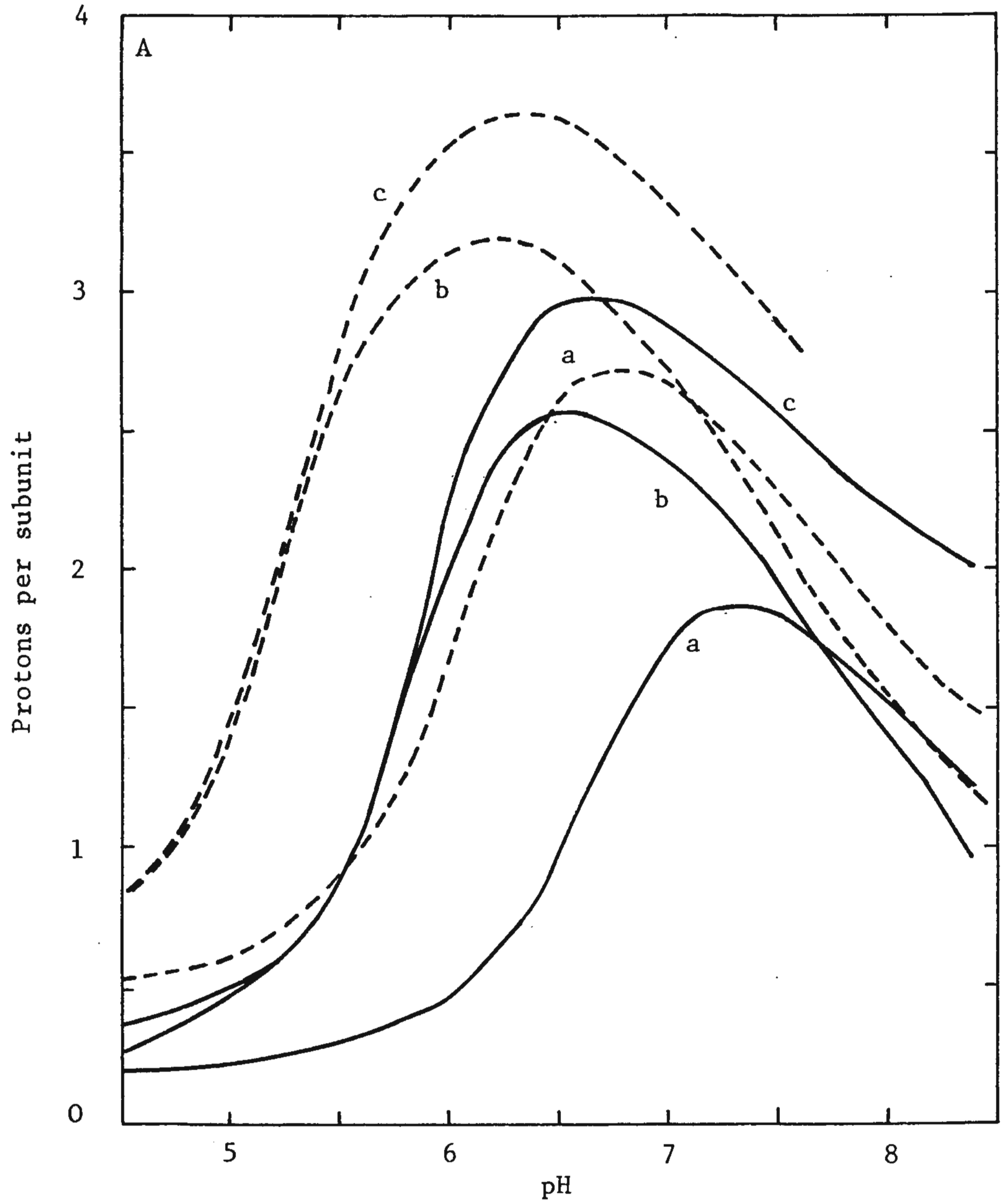


Figure 23. The displacement of protons from the U2 strain of TMV by divalent cations. The differential curves, combined for comparison, illustrate the number of protons displaced as a function of pH from the U2 strain of TMV by 1.5 mM concentrations (solid lines) and 27 mM concentrations (dotted lines) of Mg^{2+} (a), Ca^{2+} (b) and Mn^{2+} (c).

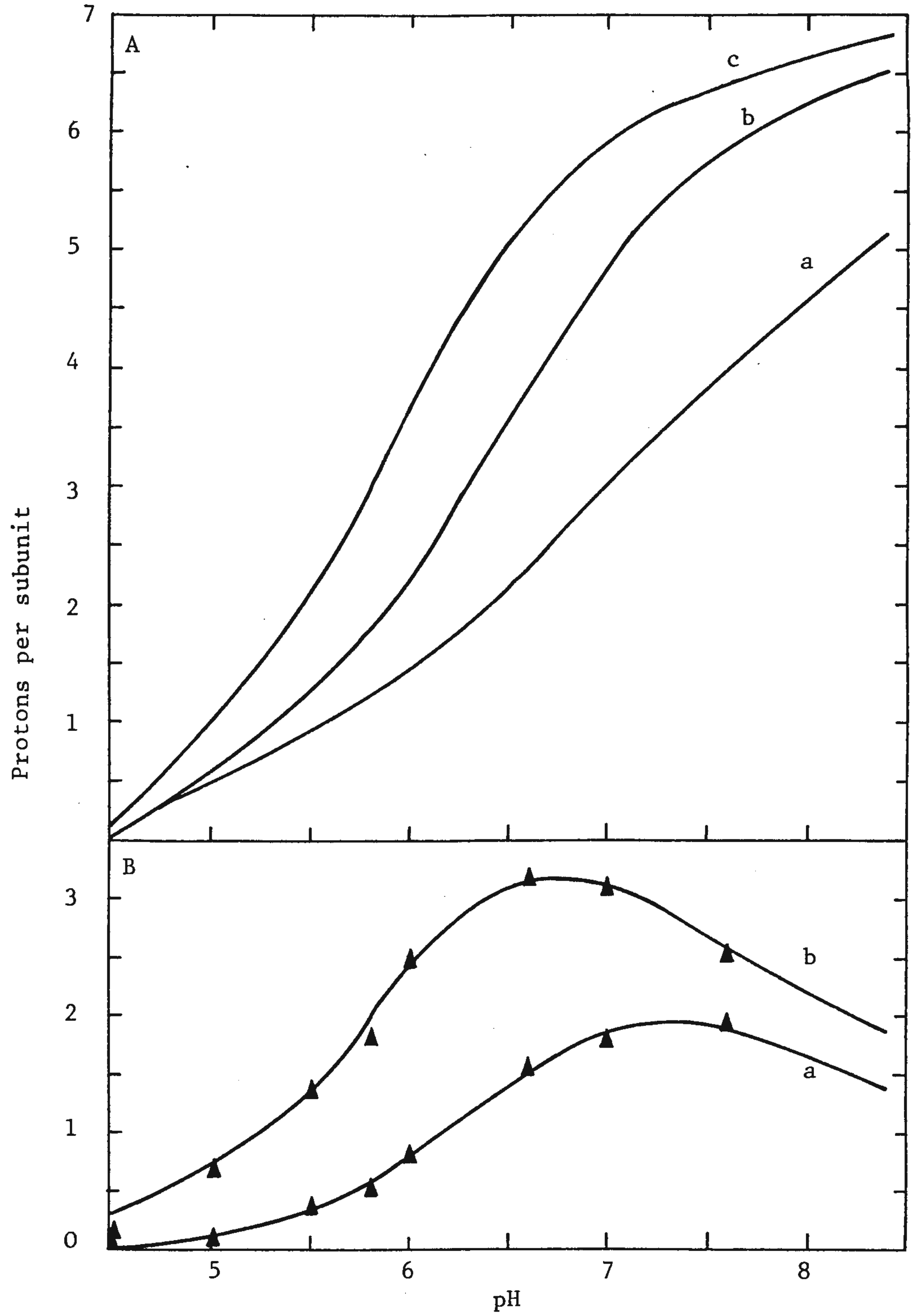


Figure 24. A. Titration curves of the cowpea strain of TMV in the presence of calcium. Cowpea strain was titrated alone (a), and in the presence of 1.5 mM Ca^{2+} (b) and 27 mM Ca^{2+} (c). The curves were positioned vertically using displacement values obtained by Ca^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from the cowpea strain of TMV by 1.5 mM (a) and 27 mM (b) Ca^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 24(A) and were accurately positioned by means of displacement values obtained by addition of calcium to aliquots of the cowpea strain of TMV (triangles).

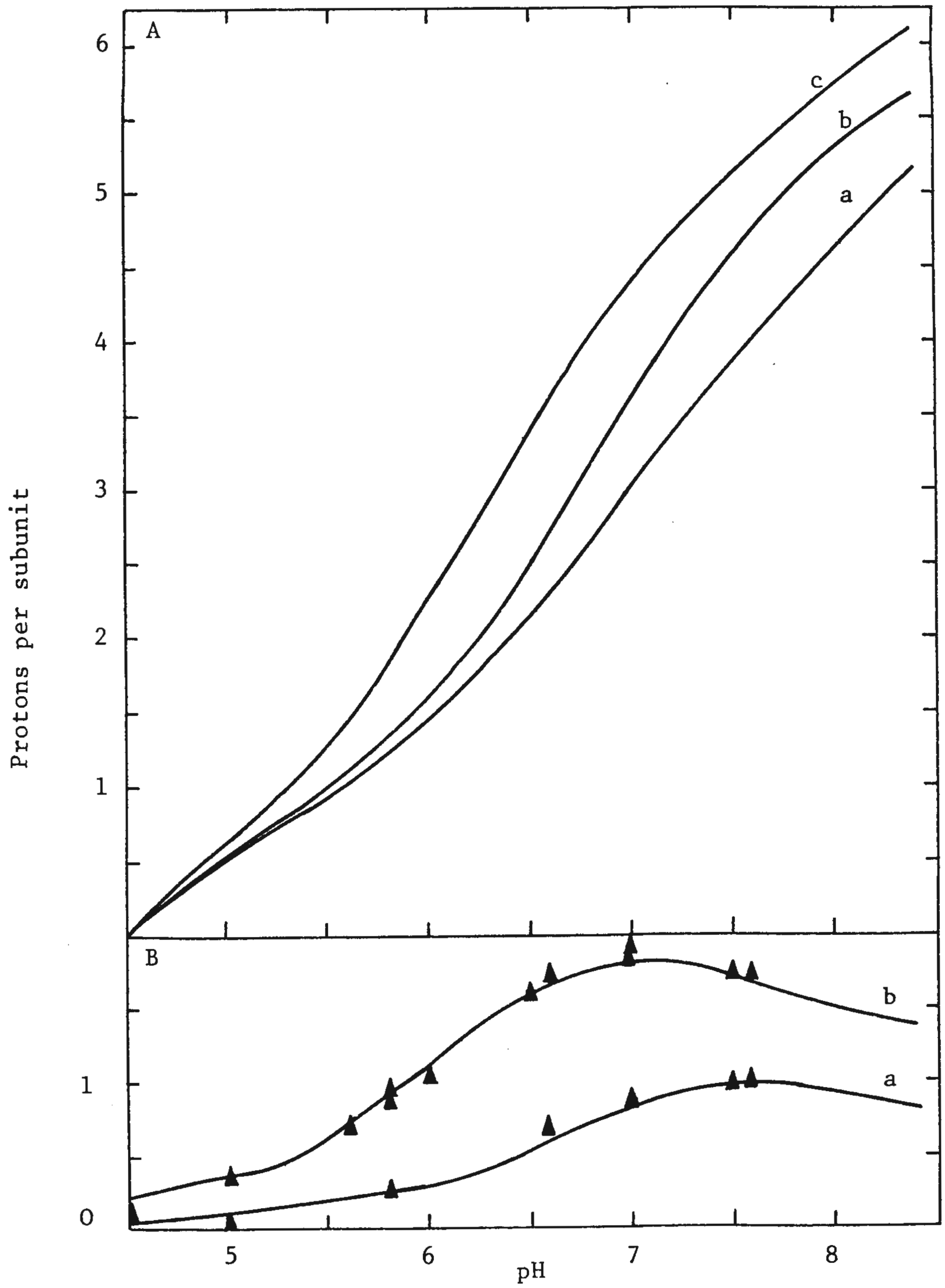


Figure 25. A. Titration curves of cowpea strain of TMV in the presence of magnesium. The virus was titrated alone (a), and in the presence of 1.5 mM Mg^{2+} (b) and 27 mM Mg^{2+} (c). The curves were positioned vertically using displacement values obtained by Mg^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from cowpea strain by 1.5 mM (a) and 27 mM (b) Mg^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 25(A) and were accurately positioned by means of displacement values obtained by addition of magnesium to aliquots of the virus (triangles).

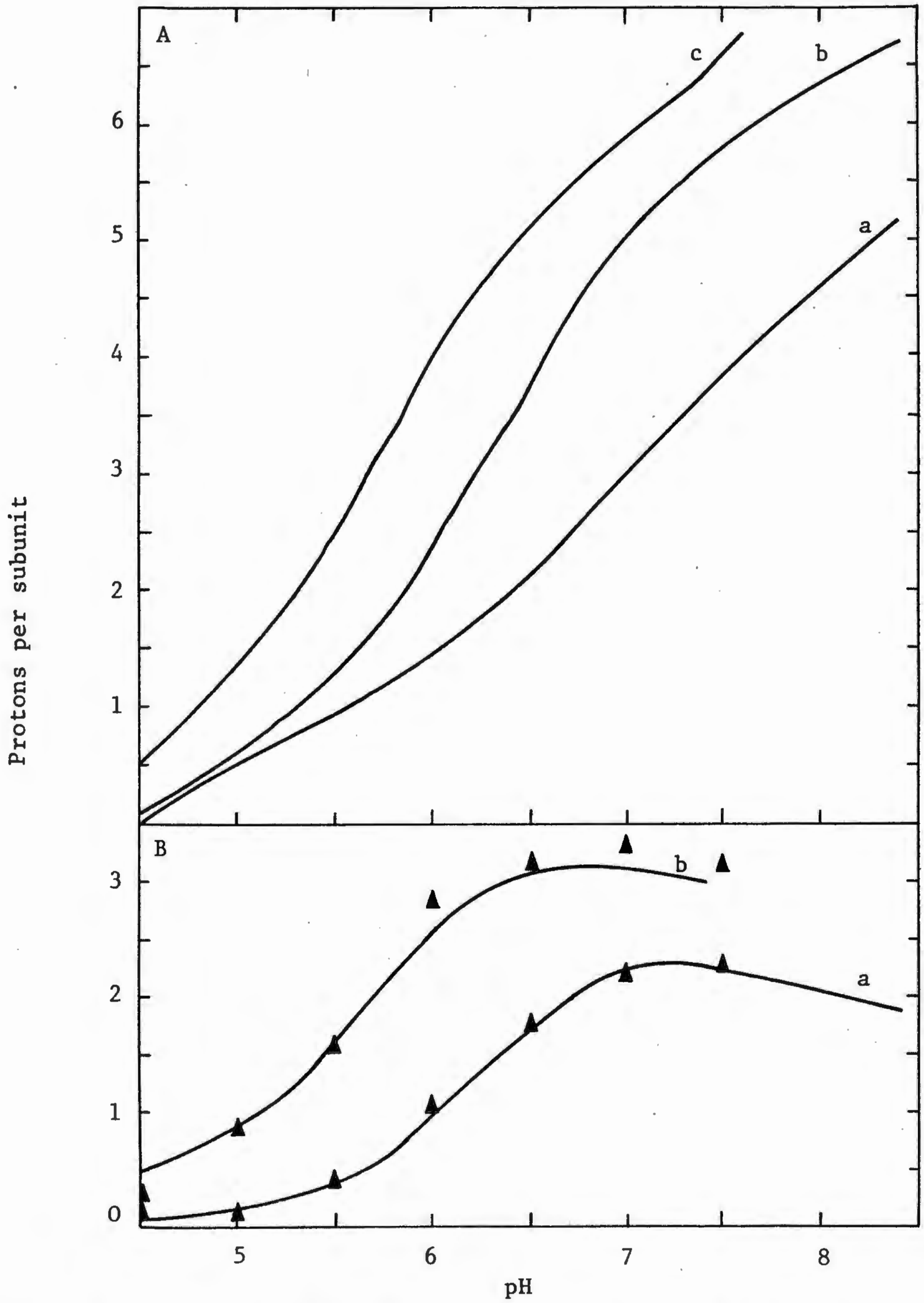


Figure 26. A. Titration curves of the cowpea strain of TMV in the presence of manganese. The virus was titrated alone (a), and in the presence of 1.5 mM Mn^{2+} (b) and 27 mM Mn^{2+} (c). The curves were positioned vertically using displacement values obtained by Mn^{2+} addition at pH 4.5.

B. Differential curves illustrating the number of protons displaced from the cowpea strain by 1.5 mM (a) and 27 mM (b) Mn^{2+} as a function of pH. The smooth curves were derived from the data in Fig. 26(A) and were accurately positioned by means of displacement values obtained by addition of manganese to aliquots of the virus (triangles).

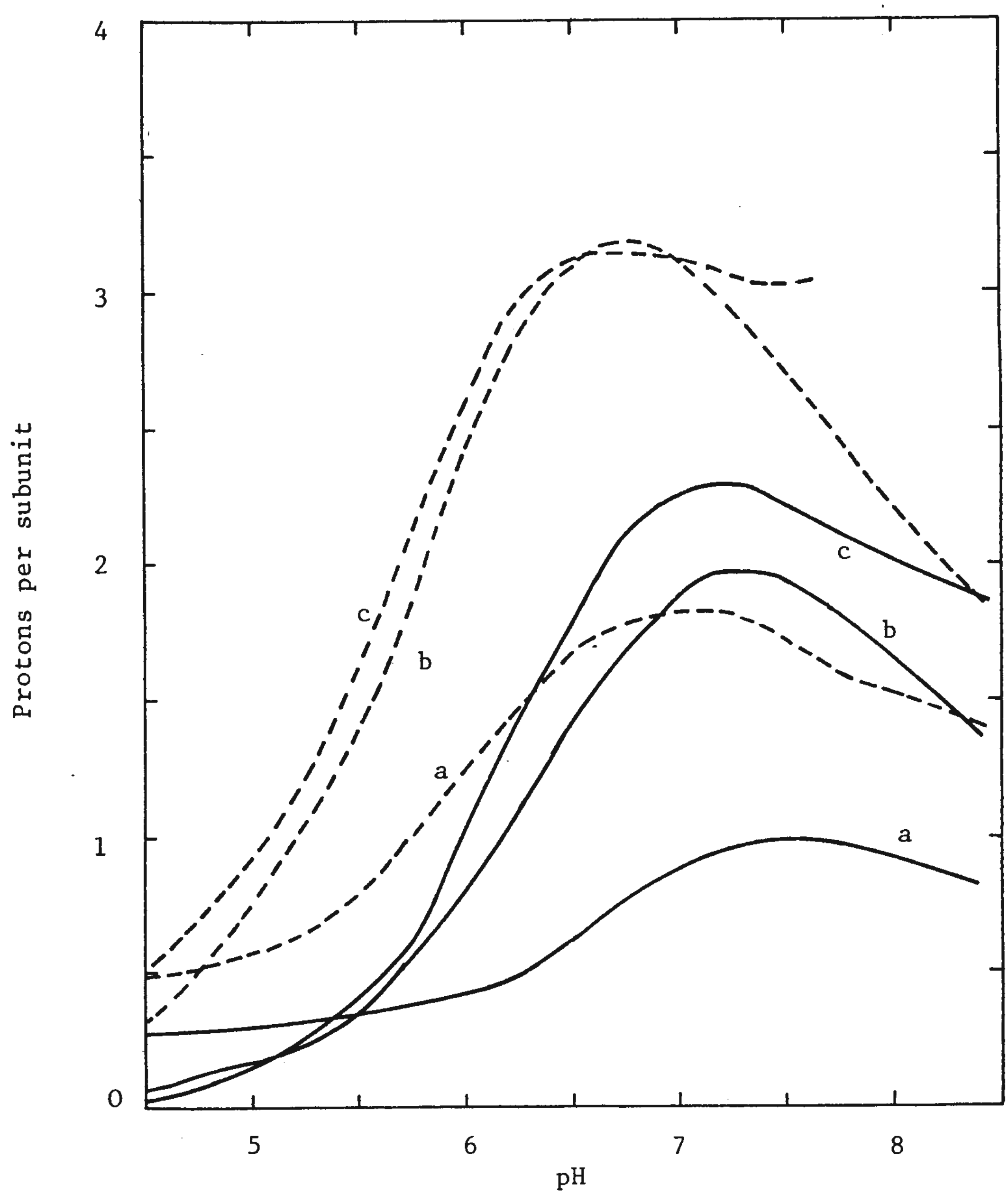


Figure 27. The displacement of protons from the cowpea strain of TMV by divalent cations. The differential curves, combined for comparison, illustrate the number of protons displaced as a function of pH from the cowpea strain of TMV by 1.5 mM concentrations (solid lines) and 27 mM concentrations (dotted lines) of Mg^{2+} (a), Ca^{2+} (b) and Mn^{2+} (c).

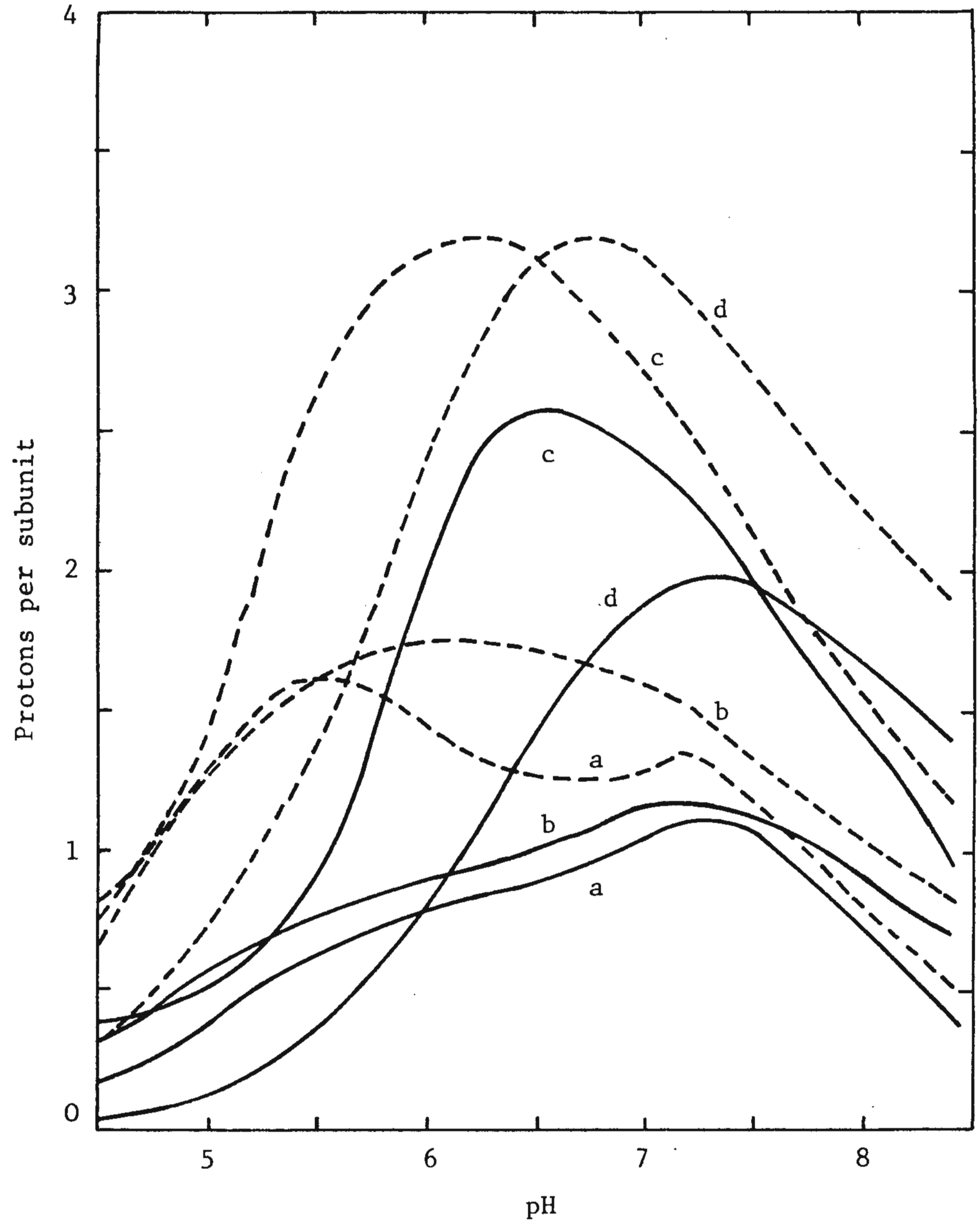


Figure 28. Proton displacement from the four TMV strains by calcium. The curves illustrate the number of protons displaced as a function of pH by 1.5 mM calcium (solid lines) and 27 mM calcium (dotted lines) from type strain (a), Y-TAMV (b), U2 strain (c) and cowpea strain (d).

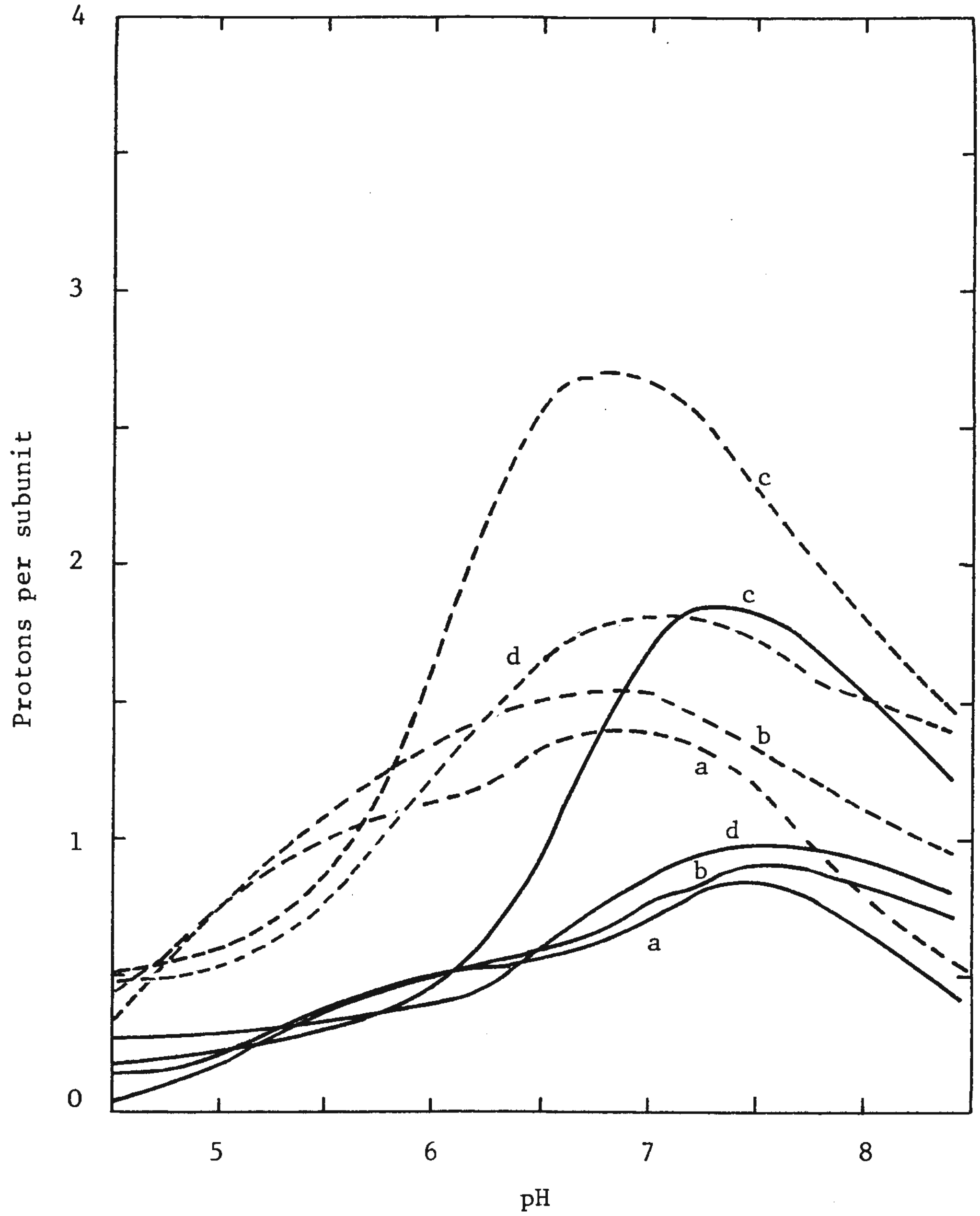


Figure 29. Proton displacement from the four TMV strains by magnesium. The curves illustrate the number of protons displaced as a function of pH by 1.5 mM magnesium (solid lines) and 27 mM magnesium (dotted lines) from type strain (a), Y-TAMV (b), U2 strain (c) and cowpea strain (d).

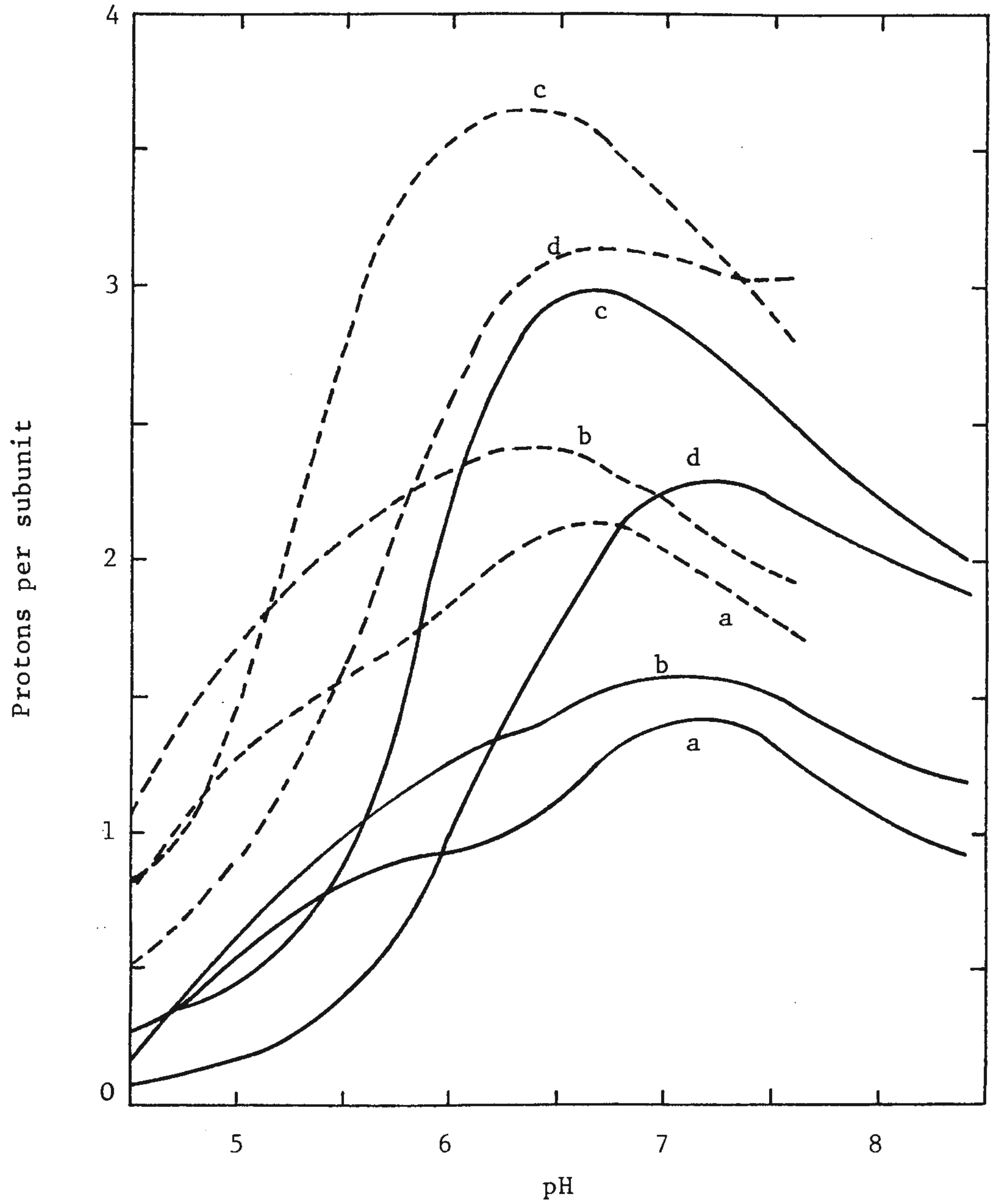


Figure 30. Proton displacement from the four TMV strains by manganese. The curves illustrate the number of protons displaced as a function of pH by 1.5 mM manganese (solid lines) and 27 mM manganese (dotted lines) from type strain (a), Y-TAMV (b), U2 strain (c) and cowpea strain (d).

(d) Discussion

The titration curve of divalent cation-free Y-TAMV (which has an identical amino acid sequence to that of the dahlemense strain except for a single serine to asparagine substitution at position 29 (Funatsu, 1964)) was very similar to that of type strain, with a total of respectively 4.8 and 5.0 protons per subunit titrating between pH 4.5 and pH 8.5. Of the prototropic residues that titrate in this pH range, Y-TAMV possesses only one more tyrosine at position 67 than type strain (see Table 2). Having an expected pK_H of 9.6, this tyrosine would only slightly have affected the titration curve which extended as far as pH 8.5. This similarity between the two strains is also seen in the similar electrophoretic mobility curves of type strain and dahlemense (Kramer & Wittmann, 1958), the slight difference between them being ascribed to the loss of a guanidyl group at position 141 in dahlemense (van Regenmortel, 1972). Thus, as discussed for type strain, Y-TAMV must also possess three abnormally titrating groups per subunit.

Proton displacement from Y-TAMV by the three cations was very similar to the corresponding displacements from type strain (Figs 28, 29 and 30), except for the slightly larger displacement from Y-TAMV in each case. In particular the markedly higher displacements at pH 6.5 by 27 mM Ca^{2+}, which amounted to an increase at pH 6.5 of nearly 0.5 protons per subunit over that of vulgare, could have been due to the pK_H 7.5 group in Y-TAMV having a greater affinity for Ca^{2+} than the corresponding group in vulgare. This group has a computed pK_{Ca} of 1.5 in the case of vulgare and, at a calcium concentration of 1.5 mM (i.e. a pCa of 2.82, so that $pK_{Ca} - pCa \simeq -1$) very little proton displacement occurs (Fig. 14, lowest curve). A ten-fold increase in the binding affinity, such that $pK_{Ca} - pCa \simeq 0$, will not result in significantly increased proton displacement at that Ca^{2+} concentration (Fig. 14). This would explain why the increased affinity for Ca^{2+} in the case of Y-TAMV does not result in significantly increased proton displacement by 1.5 mM Ca^{2+} relative to vulgare (curves a & b, Fig. 28).

However, in the case of vulgare at 27 mM Ca^{2+} (i.e. a pCa of 1.57), $pK_{Ca}-pCa \simeq 0$ for this binding site. A ten-fold increase in binding affinity, such that $pK_{Ca} - pCa \simeq 1$, will now result in a significant increase in proton displacement (see Fig. 14). This increase in proton displacement reaches a maximum when $pH - pK_H \simeq -1$, which accounts for the increase in proton displacement from Y-TAMV relative to vulgare reaching a maximum near pH 6.5 in the case of this group with a pK_H of 7.5. The pH of this maximum will obviously shift should the increase in pK_{Ca} be accompanied by an alteration in the pK_H of that group, a possibility ignored in the above argument.

Strictly, pCa is equal to $-\log[Ca]_{free}$, and is not equal to $-\log[Ca]_{total}$ as implied in the foregoing discussion. However, at both the calcium concentrations used, $[Ca]_{total} >> [TMVP]$, and thus $[Ca]_{bound}$ is always very small. Therefore, $[Ca]_{free} \simeq [Ca]_{total}$ and $pCa \simeq -\log[Ca]_{total}$. The arguments employing the term $pK_{Ca}-pCa$ are obviously only valid under these special conditions where $[Ca]_{total} >> [TMVP]$.

The increased proton displacement from Y-TAMV by 27 mM Ca^{2+} at pH 6.5 relative to that of vulgare can thus be accounted for by the pK_H 7.5 group in vulgare having an appreciably weaker affinity for Ca^{2+} than the corresponding group in Y-TAMV. The above arguments would also account for the small difference in proton displacement from the two strains by Mg^{2+}, for which both strains have a weaker affinity than for Ca^{2+}, and the larger difference in the case of Mn^{2+}, for which both strains have a stronger affinity than for Ca^{2+}.

The amino acid sequence of the Y-TAMV coat protein differs from that of vulgare coat protein at 28 positions, resulting in a difference of 15 amino acid residues in the gross composition (see Hennig & Wittmann, 1972). Ignoring the possibility that the increased affinity of a particular site for calcium may have been the result of a generalised structural modulation caused by amino acid substitutions in positions removed from the binding site, it should theoretically be feasible to identify the amino acid substitutions that could have been responsible for the increased affinity by being directly involved in the binding site. The pK_H 7.5 site, being one of the sites that bind lead in vulgare (see section B3 above), could thus be situated at either 25 Å or 84 Å radius (Caspar, 1956). As this site binds calcium, it would have to contain at least one carboxyl group (Kretsinger & Nelson, 1976). The substitutions occurring near an acidic amino acid at 25 Å radius are those at positions 100 (alanine → glutamine) and 101 (asparagine → serine), which are in the proximity of the conserved aspartic acid residues at positions 115 and 116 thought to be involved in the 25 Å site (Butler & Durham, 1972; Holmes et al., 1975; Champness et al., 1976). The alanine to glutamine substitution represents the acquisition of a side chain oxygen, being the ligand preferred by Ca^{2+}. The asparagine to serine substitution represents no gain in side chain oxygen atoms, and both amino acids have been found to be involved in Ca^{2+} binding sites (Kretsinger & Nelson, 1976).

At 84 Å radius the only conserved acidic residue is the glutamic acid at position 145. There are four or five amino acid exchanges near position 145 at positions 141, 142, 143 and 147, as well as a further four at positions 64, 65, 66 and 67 which are situated in a region of the polypeptide which is at a suitable radius to be in the proximity of residue 145 (Champness et al., 1976). In the absence of concrete information concerning this binding site, no further conclusions can be drawn regarding the involvement of one or more of these eight amino acids in a binding site at 84 Å radius. However, Durham et al., (1977b) suggested, in the light of the X-ray diffraction results of Stubbs et al., (1977), that all three sites on TMV were situated at low radius near the RNA binding site. Stubbs et al. (1977) reported that, as a result of protein aggregation, six carboxyl groups and two positively charged residues were thrust into close proximity in a "carboxyl cage", situated at 25 Å radius and otherwise lined with hydrophobic residues. They concluded that the anomalous pK_H values of TMV originated in this carboxyl cage. The alanine to glutamine substitution at position 100 in Y-TAMV would thrust an extra oxygen atom into this carboxyl cage and could thus result in one of the pre-existing calcium binding sites having an increased affinity. Also, the protein subunits in the Y-TAMV virion undergo a pairing interaction (Caspar & Holmes, 1969) which would alter the structure of the carboxyl cage, and possibly also result in the facilitated access of cations to the interior of the particle.

Supporting evidence for the cation binding sites in vulgare and Y-TAMV being in the carboxyl cage is provided by the fact that neither of the isolated proteins of the above two strains bind significant amounts of calcium (Shalaby et al., 1968; also sections D1 and D2 below). The formation of the carboxyl cage depends on both RNA presence and protein aggregation (Stubbs et al., 1977), whereas a binding site situated at 84 Å radius would not be expected to be influenced either by RNA presence or the aggregation state of the protein, and should thus bind cations both when the protein is assembled into the virus and when it is in the disaggregated state.

The titration curve of divalent cation-free U2 indicated that this strain had at least one fewer proton titrating per subunit between pH 4.5 and pH 8.5 than did vulgare. Both vulgare and U2 possess 16 titratable carboxyl groups (Hennig & Wittmann, 1972; Paulsen, 1972), which groups are the predominant ones titrating over the above-mentioned range. In addition, U2 possesses four ionisable tyrosine residues whereas vulgare possesses two (Paulsen, 1972), although this could only have influenced the upper region of the U2 titration curve. The one fewer proton titrating in the case of U2 could be explained by an additional carboxyl group relative to vulgare either titrating well below pH 4.5 (e.g. near pH 2.7, as suggested by Durham et al. (1977b) for ten carboxyl groups of vulgare) or having an anomalous pK_H even higher than the 7.1 found for vulgare, as suggested for U2 by Butler & Durham (1972). As neither the U2 nor the vulgare curves originated at their acid endpoints, they do not represent the relative degrees of protonation of the two strains at each pH.

The cation binding behaviour of U2 differed markedly from that of type strain (Figs 28, 29 & 30; also section B2 above). Both calcium and manganese, at 1.5 mM and 27 mM concentrations, displaced about 1.5 more protons per subunit from U2 than did the corresponding concentrations of that cation from vulgare (Table 13). As was found in the case of vulgare and Y-TAMV, of the three cations U2 had the weakest affinity for magnesium. The fact that well over three protons per subunit could be displaced from U2 by Ca^{2+} and Mn^{2+} ions indicated that this strain contained four cation binding sites per proton subunit, i.e. one more than vulgare.

The amino acid sequence of U2 protein differs from that of vulgare protein at 39 positions (Wittmann-Liebold & Wittmann, 1967), resulting in a net exchange of 16 amino acid residues. The three-dimensional structure of the U2 polypeptide appears, however, to be similar to that of vulgare, especially at radii greater than 70 Å (Holmes et al., 1975). The innermost regions of their protein molecules (the "vertical helix" of Stubbs et al.,

1977) do not correspond, the vertical helix being virtually absent in U2. This is ascribed to the presence of a proline residue at position 107 in U2 (Stubbs et al., 1977). Apart from not having an arginine at position 112, U2 possesses all the other residues that constitute the "carboxyl cage" of Stubbs et al. (1977); in fact, U2 and vulgare have virtually identical sequences from residue 87 to residue 122, which includes all the innermost region located at radii of less than about 40 Å in the virus particle. It is feasible that access of divalent cations to the "carboxyl cage" could be facilitated in the case of U2 by the absence of both the vertical helix and the positive charge on residue 112.

A fourth binding site is necessary, however, in addition to any possible facilitated access to the carboxyl cage, in order to explain the increased binding shown by U2. This site could also be at or near 24 Å radius, but this situation is unlikely in view of the similarity between the amino acid sequences of the U2 and vulgare polypeptides between residues 87 and 122. Isolated U2 protein (U2P) is able to bind calcium (see section D2 below): this cation displaces one more proton per subunit from U2P than from TMVP. If U2 possesses a structure similar to the "carboxyl cage" of vulgare, this would be absent in the protein (Stubbs et al., 1977; Champness et al., 1976). Thus, U2 must possess an additional binding site situated closer to the surface of the virus particle, which site is also present on the polymerised protein.

The titration curve of divalent cation-free cowpea strain had a similar shape to that of U2, the curve showing a flattening below pH 6, and becoming noticeably steeper above pH 6. This strain has sixteen potentially titratable carboxyl groups, but possesses in addition a histidine residue at position 123 (Rees & Short, 1975). Although it is possible that in the virus this residue is buried in the hydrophobic interior and is thus not available for titration, having an expected pK_H of 6.3 (see Table 1), ionisation of this residue would be expected to contribute to the steepness of the cowpea titration curve above pH 6.5, and to the increased number of titratable protons of cowpea strain relative to vulgare above pH 6.5. Cowpea strain also possesses 8 tyrosine residues, but in the absence of information as to which of these are buried, their contribution to the titration curve below pH 8.5 cannot be calculated. The amino acid sequence of the cowpea strain polypeptide differs considerably from that of vulgare. In addition to the insertion of a glutamic acid residue between positions 64 and 65, cowpea strain has two additional amino acid residues at the carboxyl end, making a total of 161 residues in the polypeptide (Rees & Short, 1975). The amino acid sequence of the cowpea strain polypeptide differs from that of vulgare at 91 positions, resulting in a net exchange of 14 amino acid residues in the gross composition.

The displacement of protons from cowpea strain by calcium and manganese was very similar to that from U2, with a maximum of over three protons per subunit being displaced by each cation (Figs 28 & 30). However, the maximum displacements in the case of cowpea strain occurred at higher pH values than was observed with U2, indicating that the sites on cowpea binding calcium and manganese had a lower affinity for these cations than the sites on U2, or that these sites on cowpea had higher pK_H values (see Fig. 14). The fact that fewer protons were displaced by 1.5 mM concentrations of each cation from cowpea strain than from U2 points to the former possibility. However, cowpea strain was similar to U2 in also possessing four sites able to bind calcium and manganese.

The binding of magnesium by cowpea strain was similar to the binding of that cation by vulgare and Y-TAMV (Fig. 29). In this respect, cowpea strain had a markedly lower affinity for Mg^{2+} ions than did U2. The slow reactivity of Mg^{2+} ions with cowpea strain in the region of pH 5.8 indicates that a site with a pK_H of about 6 offers restricted access to this cation. As proton displacement by calcium and manganese at this pH was complete within a minute or two, access of these cations was not being restricted. This site could possibly be situated in the interior of the virus, where the large hydrated diameter of magnesium ions (Williams, 1970, 1971b) prevents them from gaining access to the site.

The large number of amino acid substitutions in cowpea strain relative to vulgare makes it highly probable that their polypeptides are not identically folded. However, between positions 88 and 121 there are only 9 positions in cowpea strain that differ from vulgare, i.e. half the average substitution rate for cowpea strain. In addition, except for an aspartic acid to asparagine substitution at position 109 in cowpea strain, all the charged amino acids that constitute the "carboxyl cage" in vulgare are present in cowpea strain. One can thus infer that the folding of the cowpea strain polypeptide at radii less than 40 Å is similar to that of vulgare, and that cowpea strain also possesses multiple cation binding sites in the interior of the particle, as discussed for Y-TAMV and U2. As in the case of U2, the fourth site is probably situated at a greater radius, as the isolated cowpea strain protein is able to bind calcium, this cation displacing 1.5 protons per subunit near pH 6.7 (see section D2 below).

Proton displacement from the four TMV strains by calcium (Fig. 28) should be compared to the extents of calcium binding by each strain as determined by sedimentation (Table 2). A pCa of 2.7 is equal to a concentration of 2 mM, while 1.5 mM was the lower calcium concentration used during the titrations. At pH 6.0, the order of number of calcium ions bound is cowpea strain ≃ vulgare < Y-TAMV < U2, which is the same order as that of extent of proton displacement. In particular, U2 binds twice as many calcium ions as any of the other three strains, and calcium displaces twice as many protons from U2 as from any of the others. At pH 7.0, the corresponding order for both phenomena is vulgare < Y-TAMV < cowpea strain < U2. The sedimentation results at pH 8.0 are suspect (as discussed in section B2), but, except for the U2 result, the sequence obtained of extent of calcium binding is the same as that of extent of proton displacement.

The phenomenon of cation-binding by TMV is thus not limited to the type strain, but is shown by Y-TAMV, U2 and the cowpea strains as well. Cation binding by these three strains has a number of similarities to that by vulgare: maximum proton displacement occurs between pH 5.5 and pH 7.0; of the three cations, Mn^{2+} ions are able to displace the greater number of protons and Mg^{2+} ions the least; Mn^{2+} ions displace protons maximally near pH 6 and Mg^{2+} ions maximally near pH 7; and, the affinity for Ca^{2+} ions is significantly greater than that for Mg^{2+} ions. However, the greater displacement of protons by these cations from the U2 and cowpea strains relative to that from vulgare and Y-TAMV indicates the presence of an additional cation-binding site in the former two strains. The precise localisation of the binding sites in the virions of each strain is not yet possible. In no instance was the affinity of Y-TAMV, U2 and cowpea strains for these cations weaker than that of vulgare. Thus, as discussed for vulgare, dissociation of calcium from the virion could feasibly play a role in *in vivo* disassembly of these three strains as well.

D. The proteins of the four TMV strains

The binding of divalent cations by TMVP was investigated in the present study for two reasons. Firstly, at least one of the cation-binding sites on TMV being thought to involve the RNA (see section A1(c) of Chapter 2), the cation-binding ability of TMVP was compared with that of TMV. Residual binding of cations by TMVP would confirm that RNA-independent cation binding was occurring, whereas an absence of cation binding by TMVP, particularly when in the helical form, would indicate that binding requires RNA. Also, as discussed in section D of Chapter 2, TMVP would be expected to have a lower affinity than TMV for divalent cations if their dissociation from TMV is a prerequisite for virus disassembly. Secondly, it was hoped that this investigation would indicate whether the cation-induced stabilisation of polymerised TMVP (McMichael & Lauffer, 1975) was in any way related to the cation-induced stabilisation of the TMV virion (Brakke & van Pelt, 1969; Powell, 1975).

1. Direct measurement of calcium bound to TMVP

Calcium binding to TMVP was measured by equilibrium dialysis in a similar manner to that done with TMV (see section B2 of Chapter 3). TMVP bound very little calcium between pH 5.5 and pH 7.8 at pCa values of 2.7 and 3.0 (Table 14).

TABLE 14

The binding of calcium by tobacco mosaic virus protein

pCa	pH	Ca^{2+} ions bound per TMVP subunit
3.0	6.23	0.03
3.0	6.27	0.06
3.0	6.32	0.08
3.0	6.49	0.08
3.0	6.57	0.08
3.0	7.77	0.05
2.7	5.50	0.02
2.7	6.25	0.08
2.7	7.77	0.11

2. Titration of the proteins of the four TMV strains

(a) Titration of type strain protein

Type strain protein (TMVP) that had been freed of divalent cations was titrated in 50 mM KCl at concentrations ranging from 5 to 10 mg/ml. Samples to be used for titration, kept initially at $4^{o}C$ in 50 mM KCl at pH 5, were allowed to equilibrate to room temperature ($21^{o}C$) for a few hours prior to titration. The titration curve (curve a, Fig. 31A) was found to be reproducible from batch to batch, and was characterised by a hysteresis loop equivalent to a maximum of 0.5 protons per subunit at pH 6.2. A total of 4.0 protons per subunit titrated between pH 4.5 and pH 8.5, i.e. one fewer than the number that titrated over the same pH range in the case of intact TMV (curve a, Fig. 6A). TMVP appeared to have lost a group which in intact TMV titrated above pH 7, as, between pH 6.7 and pH 8.5, in the virus 2.0 protons per subunit titrated (see curve a, Fig. 6A) as opposed to only one in the protein. Between pH 4.5 and pH 6.7, 3 protons per subunit titrated in both cases.

A feature of the protein titration was the tendency of the pH readings to drift upwards for up to 30 minutes after the addition of titrant. This occurred near pH 7.0 on both branches of the titration curve, and near pH 6.0 on the descending branch. A protein titration between pH 4.5 and pH 8.5 consequently exceeded 3 hours in one direction. Virus titrations were characterised by rapid attainment of equilibrium and seldom took more than one hour.

Titration of TMVP in the presence of 1.5 mM Ca^{2+} (curve b, Fig. 31A) showed a slightly diminished hysteresis at pH 6.2. The titration curve was very similar to that obtained in the absence of calcium, displacement of protons by the Ca^{2+} nowhere exceeding 0.1 protons per subunit.

The addition of calcium to 27 mM final concentration virtually abolished the titration hysteresis at pH 6.2 (curve c, Fig. 31A). The titration curve resembled the downward branch of the curve obtained in Ca^{2+} absence, indicating that addition of Ca^{2+} to 27 mM concentration at pH 6.2 during the upward titration resulted in the displacement of 0.5 protons per subunit (curve b, Fig. 31B). A further effect of Ca^{2+} at 27 mM concentration was the flattening of the shoulder in the titration curve near pH 7.2. The presence of 27 mM Ca^{2+} largely eliminated the pH drift observed during titrations in the absence of calcium.

(b) Titration of Y-TAMV protein

The titration curve of divalent cation-free Y-TAMV protein (YP) (curve a, Fig. 32A) had a number of features in common with that of type strain protein, viz. a hysteresis loop at pH 6.2, a shoulder near pH 7.0 and one fewer proton titrating between pH 4.5 and 8.5 in the protein than in the virus. As in the case of type strain, a group titrating above pH 7 in the virus is absent in the protein: in both virus and protein 3.0 protons per subunit titrate between pH 4.5 and 6.6, whereas, between pH 6.6 and 8.5, 1.8 protons per subunit titrate in the virus but only 0.7 in the protein.

Titration of YP in the presence of 1.5 mM Ca^{2+} (curve b, Fig. 32A), showed diminished hysteresis at pH 6.2. This curve tended to resemble the downward branch of the curve obtained in the absence of calcium ions. The presence of 1.5 mM Ca^{2+} during the upward titration resulted in proton displacement which reached a maximum of 0.25 protons per subunit at pH 6.2 (curve a, Fig. 32B). This amount of Ca^{2+} induced proton consumption (i.e. a rise in pH) by YP between pH 6.5 and pH 7.0.

Titration of YP in the presence of 27 mM calcium (curve c, Fig. 32A) showed no hysteresis, and resulted in a curve without a shoulder at pH 7. Calcium at this concentration resulted in proton displacement, with a maximum at pH 6.0 of about 0.6 protons per subunit, and induced proton consumption below pH 5.0 and between pH 6.5 and pH 8.0 (curve b, Fig. 32B). Maximum proton consumption of 0.5 protons per subunit occurred at pH 6.8.

(c) Titration of the protein of U2 strain

The titration curve of divalent cation-free U2 protein (U2P) (curve a, Fig. 33A) contained a hysteresis loop centered on pH 6.2, where the hysteresis amounted to a maximum of 0.5 protons per subunit, as well as a shoulder at pH 7. A total of 3.9 protons per subunit titrated between pH 4.5 and pH 8.5, whereas in the virus (curve a, Fig. 20A) 3.8 protons per subunit titrated over the same pH range. However, below pH 7.0, 3.6 protons titrated in the protein while only 2.2 titrated in the virus. The protein thus has a markedly increased buffering capacity below pH 7.0 relative to that of the intact virus.

Titration of U2P in the presence of 1.5 mM Ca^{2+} (curve b, Fig. 33A) showed a total absence of hysteresis. Significant proton displacement by the Ca^{2+} occurred between pH 5 and pH 7, with a maximum of 1.1 protons per subunit being displaced at pH 6.3 (Fig. 33B).

In the presence of 27 mM Ca^{2+}, U2P showed no titration hysteresis (curve c, Fig. 33A), and proton displacement occurred between pH 4.8 and 7.5 (Fig. 33B). A maximum of 1.7 protons per subunit were displaced at pH 6.0. Calcium-induced proton consumption by the protein was observed only below pH 4.8.

(d) Titration of the protein of cowpea strain

The titration curve of divalent cation-free cowpea protein (CP) (curve a, Fig. 34A) differed from that of the other three strains in containing no hysteresis loop. This feature was found to be reproducible using different batches of protein. Between pH 4.5 and pH 8.5, a total of 4.6 protons per subunit titrated in the protein, compared with a total of 5.2 in the virus (curve a, Fig. 24A). Except for the shoulder in the protein curve at pH 7, the virus and protein titration curves showed no marked differences.

Titration of CP in the presence of 1.5 mM calcium (curve b, Fig. 34A) indicated indicated that protons were displaced by the calcium. Proton displacement occurred between pH 5.4 and pH 7.1, with a maximum of 0.6 protons per subunit being displaced at pH 6.7 (Fig. 34B). Titration in the presence of 27 mM Ca^{2+} (curve c, Fig. 34A) resulted in proton displacement above pH 5.1, with a maximum of 1.5 protons per subunit being displaced by the calcium at pH 6.6 (Fig. 34B). The calcium induced proton consumption by the protein below pH 5.1.

3. Ultracentrifugal investigation of the effect of calcium on TMVP polymerisation

Following the observations that calcium could either displace protons from or induce proton consumption by the four TMV proteins, depending upon the pH (see Results, Section D2), an attempt was made using analytical ultracentrifugation to determine whether Ca^{2+} ions influenced the equilibria between the various TMVP polymerisation states. It was hoped that those experiments would also shed light on the observation of McMichael & Lauffer (1975) that treatment of TMVP with Ca^{2+} stabilised the polymerised protein at pH 6.5.

(a) TMV (type strain) protein

The sedimentation behaviour of TMVP as a function of pH, both in the absence and the presence of calcium, is illustrated in Figure 35. In the absence of calcium, a species sedimenting at 8 S (A-protein) was the only

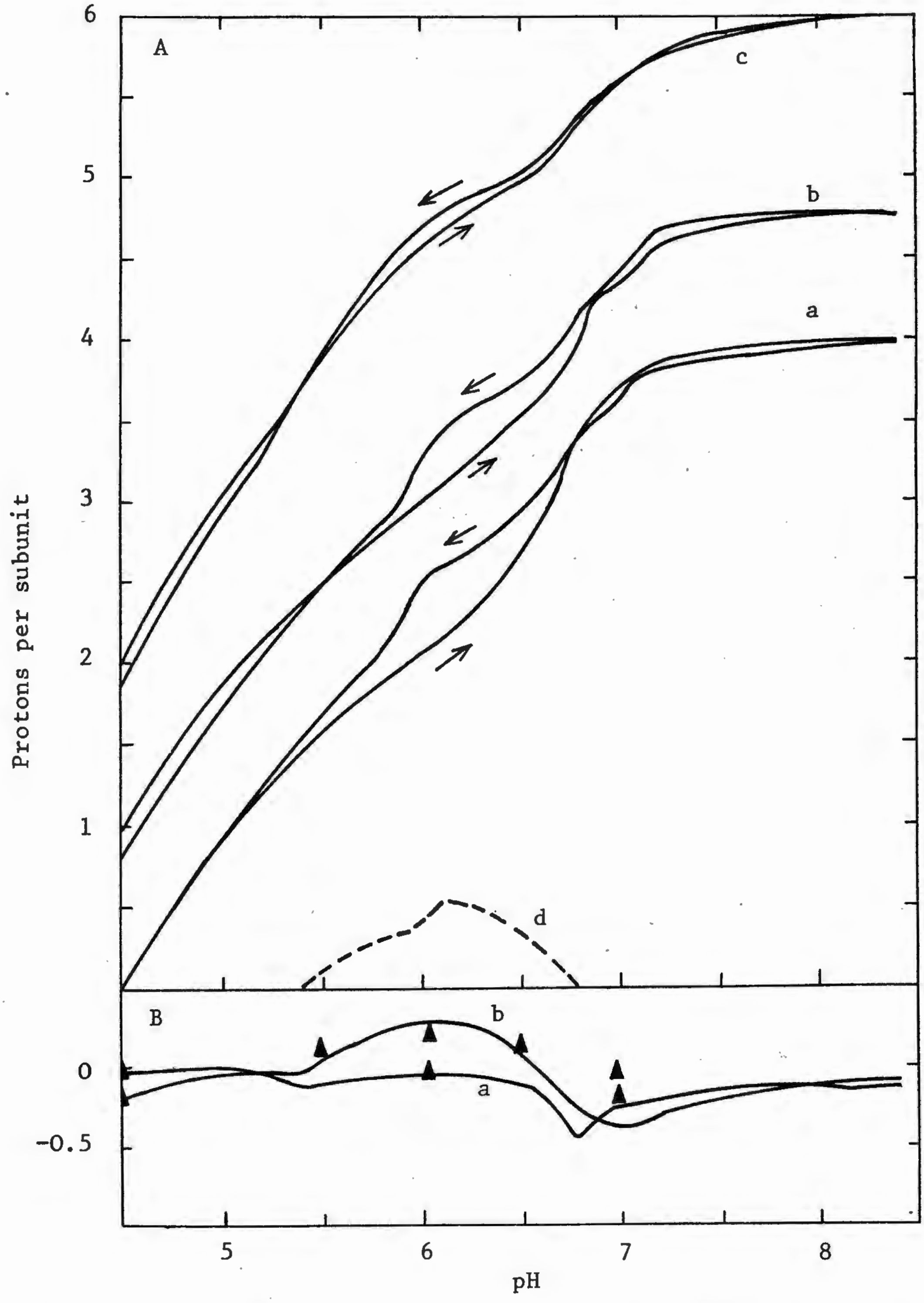


Figure 31. A. Titration curves of the protein of TMV type strain, TMVP, in the presence of calcium. Protein was titrated alone (a), and in the presence of about 1.5 mM (b) and 27 mM (c) calcium. The arrows indicate forward (alkali titrant) and reverse (acid titrant) titrations, while the extent of hysteresis in (a) is illustrated by the dotted line (d).

B. Differential curve illustrating the number of protons displaced from or consumed by TMVP as a function of pH, due to the addition of Ca^{2+} to 1.5 mM (a) and 27 mM (b) final concentration. The smooth curves were derived from the forward titration data of (A), and were accurately positioned vertically using displacement values obtained by the addition of calcium to aliquots of TMVP (triangles).

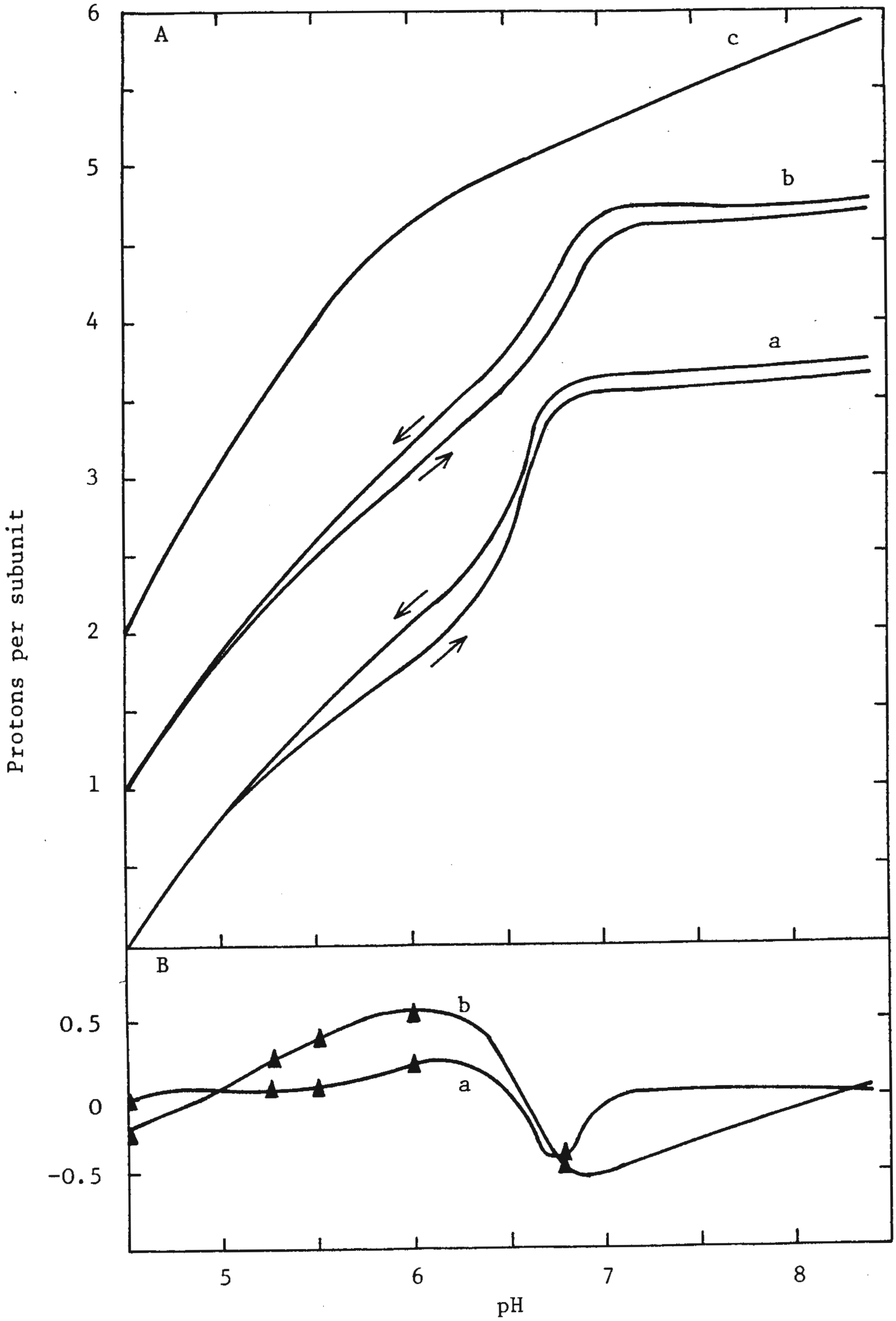


Figure 32. A. Titration curves of the protein of Y-TAMV, YP, in the presence of calcium. Protein was titrated alone (a), and in the presence of 1.5 mM (b) and 27 mM (c) calcium. The arrows indicate forward (alkali titrant) and reverse (acid titrant) titrations. Vertical positioning is arbitrary.

B. Differential curves illustrating the number of protons displaced from or consumed by YP as a function of pH, due to the addition of Ca^{2+} to 1.5 mM (a) and 27 mM (b) final concentration. The smooth curves were derived from the forward titration data of (A), and were accurately positioned vertically using displacement values obtained by addition of Ca^{2+} to aliquots of YP (triangles).

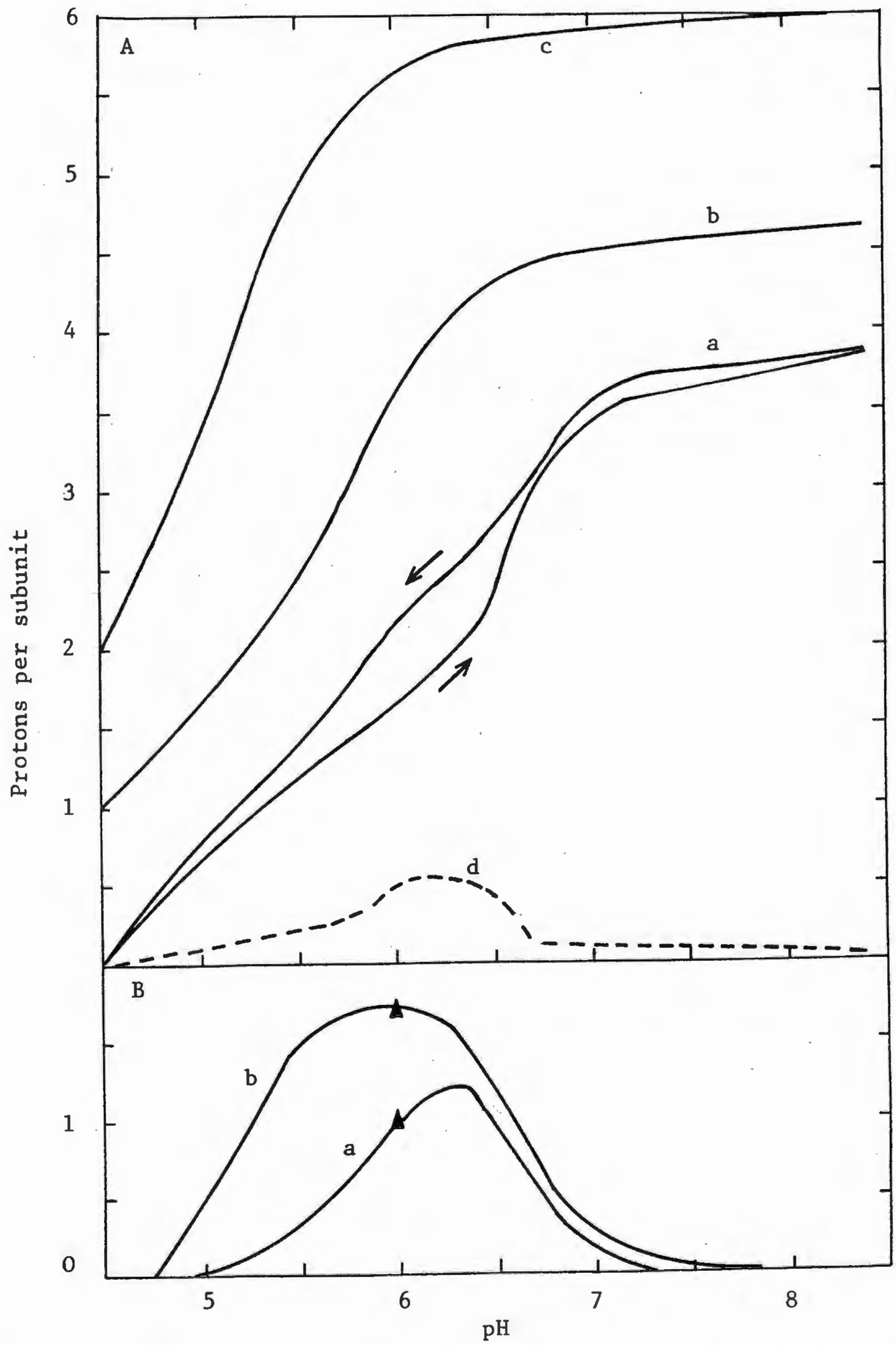


Figure 33. A. Titration curves of the protein of the U2 strain of TMV, U2P, in the presence of calcium. Protein was titrated alone (a), and in the presence of 1.5 mM (b) and 27 mM (c) calcium. The arrows indicate forward (alkali titrant) and reverse (acid titrant) titrations, while the extent of hysteresis in (a) is illustrated by the dotted line (d). Vertical positioning of (b) and (c) relative to (a) is arbitrary.

B. Differential curves illustrating the number of protons displaced from or consumed by U2P as a function of pH, due to the addition of calcium to 1.5 mM (a) and 27 mM (b) final concentration. The curves were derived from the forward titration data in (A) and were accurately positioned vertically using displacement values obtained by addition of Ca^{2+} to aliquots of U2P (triangles).

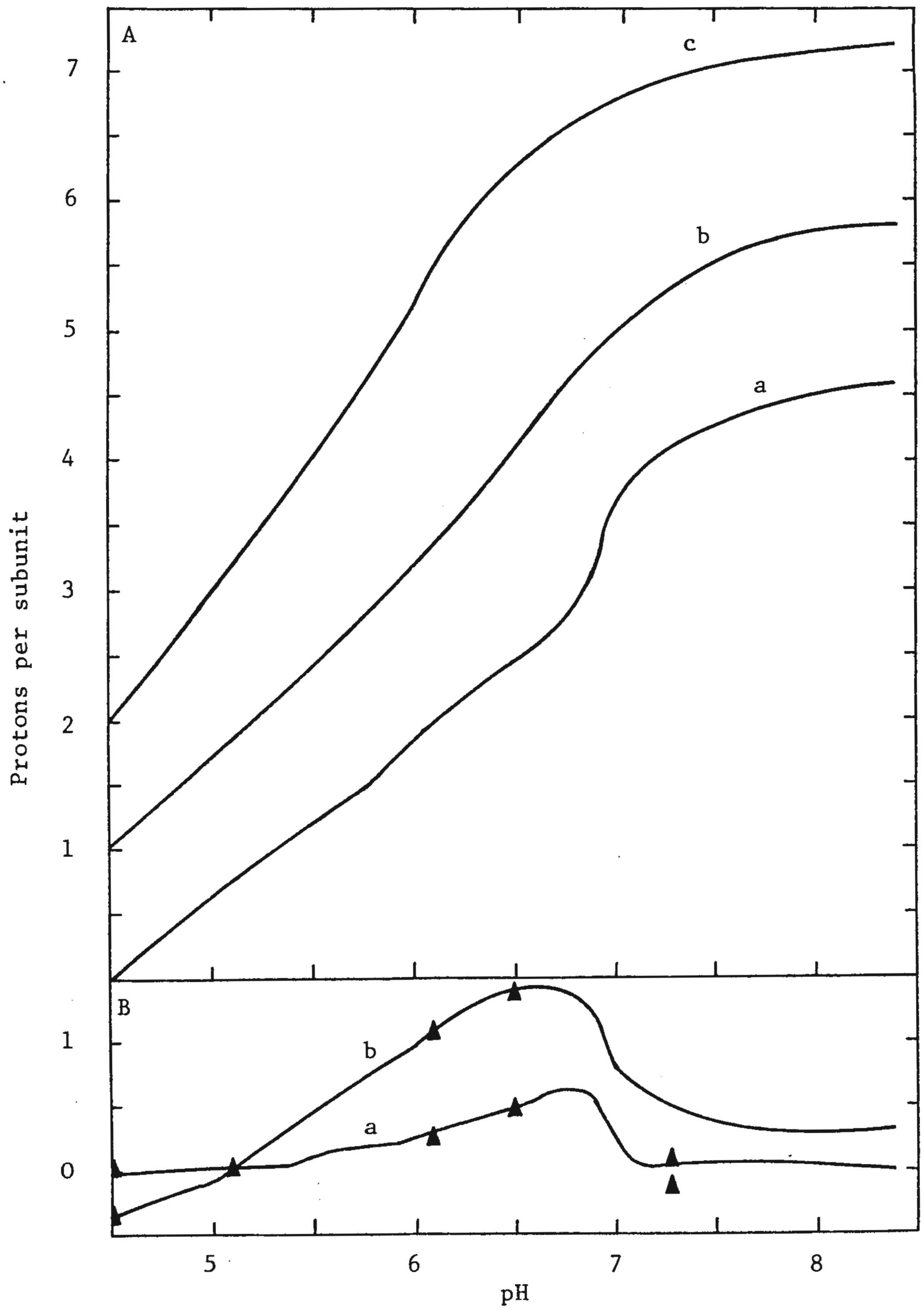


Figure 34. A. Titration curves of the protein of the cowpea strain of TMV, CP, in the presence of calcium. Protein was titrated alone (a), and in the presence of 1.5 mM Ca^{2+} (b) and 27 mM Ca^{2+} (c). Vertical positioning of (b) and (c) relative to (a) is arbitrary.

B. Differential curves illustrating the number of protons displaced from or consumed by CP as a function of pH, due to the addition of Ca^{2+} to 1.5 mM (a) and 27 mM (b) final concentration. The curves were derived from the data in (A), and were accurately positioned vertically using displacement values obtained by addition of Ca^{2+} to aliquots of CP (triangles).

species detectable at pH 7.6 and higher. At pH 7.2, a 26-28 S species (2-disk-stack) made its appearance. As the pH was lowered further, a 41 S species (possibly short helices or 3- or 4-disk-stacks) dominated at pH 6.2 and at lower pH values even higher aggregates (>100 S, presumably long protein helices) were formed.

In the presence of 40 mM calcium, the equilibrium was significantly altered in the direction of the higher aggregates. At pH 8.0, a number of species ranging from 14 S to 29 S were present in addition to the 4 S species. At pH 7.6, a 5 S and a 31 S species could be resolved, the former decreasing and the latter increasing as the pH was lowered down to pH 6.8, where a 35 S species predominated. Further aggregation occurred as the pH was lowered, a 60 S species and a 76 S species predominating at pH 6.5 and 6.2 respectively. In some TMVP preparations, protein helices (>100 S) were evident at pH 6.5 and lower, in addition to 60-70 S material. At pH 5.0 only large aggregates (>200 S) were evident.

The effect of calcium was further demonstrated if TMVP was dialysed and centrifuged at $4^{o}C$ (Fig. 36). At pH 6.5 and pH 6.2, only 3 S A-protein was present in the absence of calcium. If calcium was present, at both pH's the amount of A-protein was diminished, and at pH 6.5 and pH 6.2 a 25 S species and a 27 S species respectively were present.

A comparable effect was observed if calcium was added during the titration of TMVP (Fig. 37). During a forward titration (alkali titrant), addition of calcium induced disk formation at pH 7.2 and helix formation at pH 6.8. During a reverse titration (acid titrant), calcium addition at pH 6.2 resulted in the disappearance of both a 24 S and a 36 S species and the appearance of a 72 S species. Calcium addition at pH 6.2 during a forward titration had no noticeable effect.

The effect of calcium can thus be seen at pH 7.5 and above at 20^{o}C (and at pH 6.5 and 6.2 at 4^{o}C), where Ca^{2+} induced the formation of disks at the expense of A-protein. Also, from pH 7.2 down to pH 6.2 in the presence of Ca^{2+}, aggregates were formed having higher sedimentation coefficients than those formed in the absence of Ca^{2+}. Finally, formation of helices commenced at a higher pH in the presence of Ca^{2+} than in its absence.

(b) Y-TAMV protein

The effect of calcium on YP is shown in Figure 38. In the absence of Ca^{2+}, the aggregation behaviour of YP was very similar to that of TMVP, with a 4-6 S species being present above pH 6.5, 23-25 S and 28-38 S species being present from pH 6.5 to pH 7.0, and species >100 S being present below pH 6.8. In the presence of 40 mM Ca^{2+}, rapidly sedimenting species were present at pH 8.0, and at lower pH's. However, calcium did not appear to induce formation of 20 S disks, or stacks thereof, but induced the formation of a heterogeneous mixture of large aggregates sedimenting at about 200 S. These were present over the entire pH range from pH 7.5 down to pH 5.0, with the 3 S species diminishing with decreasing pH and being absent at pH 6.5. At pH 6.5 and pH 6.2, a species sedimenting at about 50 S was present, while at pH 5.0 only a 196 S species was detected.

The effect of Ca^{2+} addition at pH 6.8 during a titration of YP is shown in Figure 39. The 4 S and 29 S species present in calcium absence were converted to a heterogeneous mixture of aggregates sedimenting at about 140 S.

The effect of Ca^{2+} on YP was thus to favour the promotion of large aggregates, species sedimenting at 200 S (presumably large helical aggregates) being formed at pH's as high as 7.5. There was no evidence of disk formation in Ca^{2+} presence, except for the 50 S species formed at pH 6.5 and 6.2.

(c) TMV (cowpea strain) protein

The effect of Ca^{2+} on the polymerisation of CP (Fig. 40) was markedly different from the effect on the polymerisation of TMVP or YP. In the absence of Ca^{2+}, a 5 S species was present at pH 7.5 and diminished down to pH 6.8, where it was no longer present. A 22 S species was also present at pH 7.5, and its sedimentation velocity increased steadily with drop in pH, such that at pH 6.5 it sedimented at 32 S. This species was characterised by a sharp schlieren peak which broadened slowly, indicating that a discrete, homogeneous aggregate was present. At pH 6.2, the slowest species sedimented at 68 S and there was evidence of a continuum of higher order aggregates. In addition, at pH 6.2 a 178 S species made its appearance.

In the presence of 40 mM Ca^{2+}, the equilibrium was again shifted in favour of larger aggregates. At no pH between pH 7.5 and pH 6.2 was there evidence of A-protein. The discrete species, which in the absence of Ca^{2+} sedimented between pH 7.5 and pH 6.5 at 22-32 S, was converted in the presence of Ca^{2+} to a more heterogeneous mixture with a mean sedimentation velocity about 30% higher. Only at pH 6.2 was there, in addition to the major component sedimenting at 75 S, an indication of aggregates sedimenting at 100 S or more.

The effect of the addition of Ca^{2+} at pH 6.8 during a titration of CP is shown in Figure 41. Aggregates sedimenting heterogeneously at about 41 S in the absence of Ca^{2+} were converted by Ca^{2+} to a slightly less heterogeneous 50 S species.

The effect of Ca^{2+} on CP was thus to favour the formation of what are, presumably, heterogeneous mixtures of limited stacks of disks. The extent of aggregation was slightly higher than in the absence of Ca^{2+}, and increased as the medium became more acidic. Between pH 6.2 and pH 7.5, there was virtually no formation of protein helices.

(d) U2 protein

The effect of Ca^{2+} on the polymerisation of U2P (Fig. 42) is similar to the effect on the polymerisation of CP. In the absence of Ca^{2+}, a 7 S species is present at pH 7.2 and above, a 20 S species (disk) is present from pH 7.5 down to pH 6.2, and a 30 S species (perhaps a 2-disk-stack) is present at pH 6.5 and below. At pH 5.0, a 146 S species is the only one present. In the presence of 40 mM Ca^{2+}, four species of aggregate were formed, which persisted from pH 7.5 to pH 6.2, over which pH range their sedimentation velocities did not noticeably alter. The slowest species sedimented at 40-44 S, the major aggregate at 45-49 S, the next major aggregate at 52-56 S, and the fastest aggregate at 57-63 S. At no pH between pH 7.5 and pH 6.2 was there evidence of A-protein, or of large aggregates of >100 S.

The addition of Ca^{2+} to U2P during a titration (Fig. 43) produced a similar effect to that obtained by dialysis. At pH 7.2 species of 4 S and 18 S present in the absence of Ca^{2+} were converted by Ca^{2+} addition to four faster sedimenting species. The addition of Ca^{2+} at pH 6.8 converted a heterogeneous species sedimenting at about 29 S to a range of larger aggregates of which the major component sedimented at 102 S.

The addition of calcium thus promoted the aggregation of U2P. Mixtures of A-protein plus disks, or disks plus 2-stack-disks, were converted by Ca^{2+} to aggregates of presumably 3-stack-disks and higher which then each sedimented discretely. From pH 7.5 to pH 6.2 in the presence of Ca^{2+}, there was no evidence of either A-protein or of large protein helices.

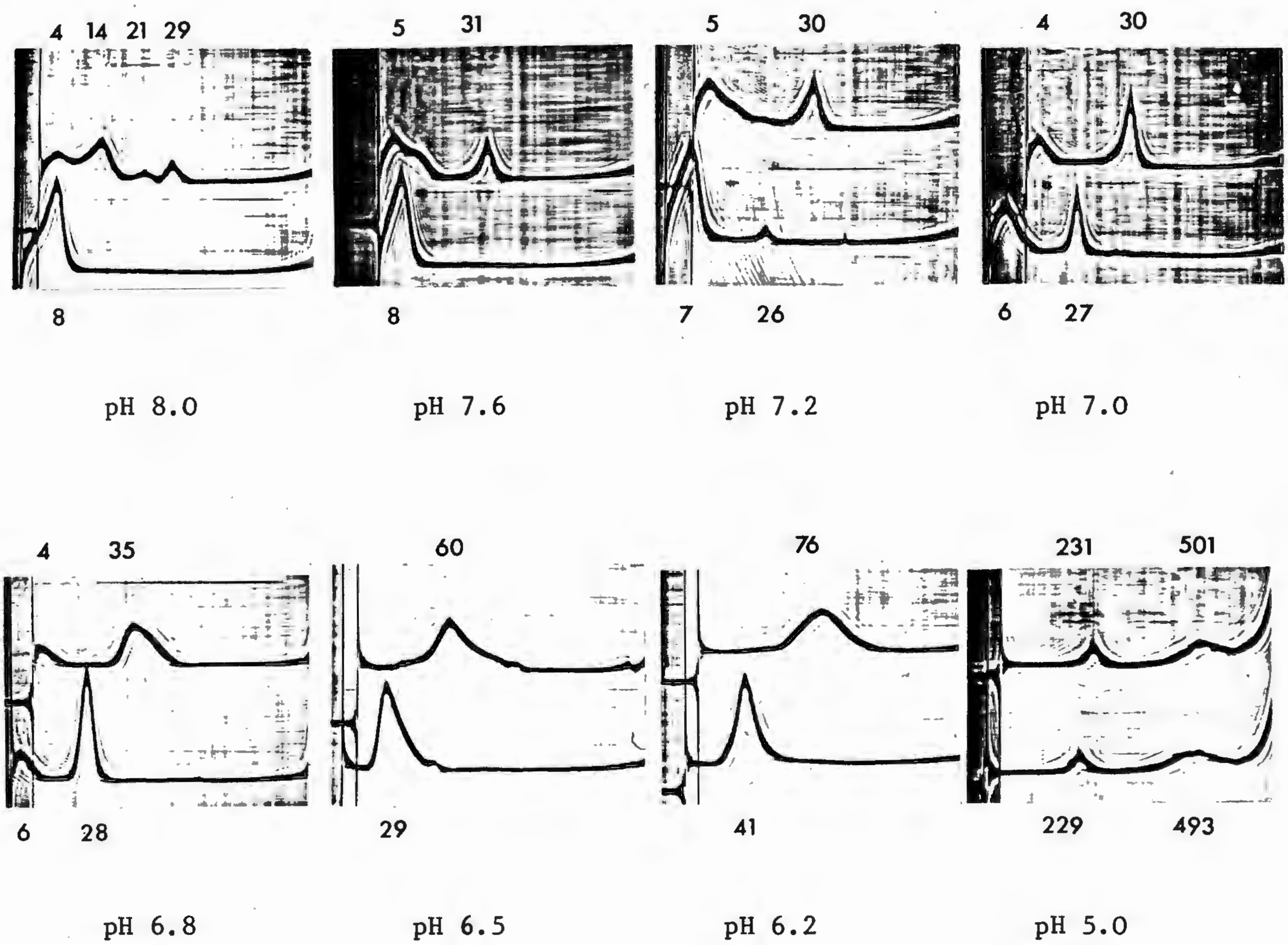
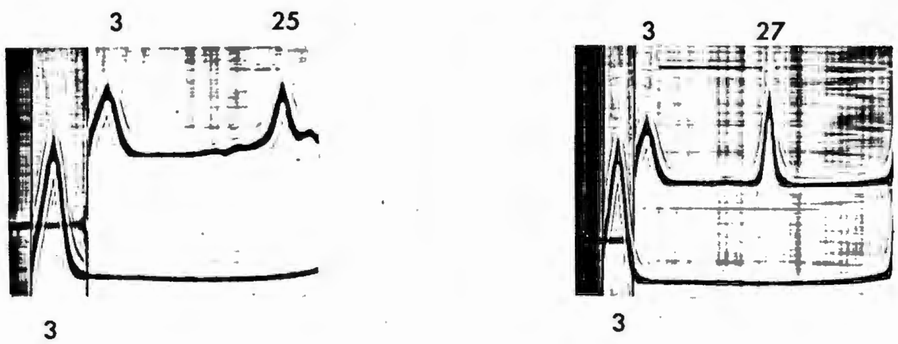
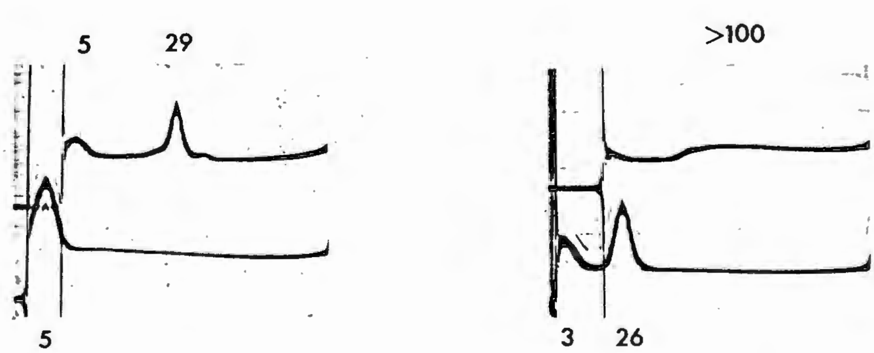


Figure 35. Sedimentation patterns of protein of TMV type strain at various pH values, all at 20^{o}C and ionic strength I=0.16. The protein, originally in 50 mM KCl at about pH 5.5, was dialysed at a concentration of 5 mg/ml for at least 48 h at 20^{o}C against 0.04-0.07 M imidazole/HCl buffers at the indicated pH value. The buffer solutions were either devoid of Ca^{2+} (lower pattern of each photograph) or contained 40 mM Ca^{2+} (upper pattern of each photograph), and the ionic strength in each case was adjusted to I=0.16 by the addition of KCl. The numbers on these and subsequent pictures represent S values. The rotational speeds, and times of centrifuging before taking each photograph, were not identical, but were chosen optimally to illustrate the protein species present.



pH 6.5 pH 6.2

Figure 36. Sedimentation patterns of protein of TMV type strain at pH 6.5 and pH 6.2, both at 4^{o}C and I=0.16. Apart from the dialysis and sedimentation having been done at 4^{o}C, all experimental details were identical to those of Fig. 35.



pH 7.2 pH 6.8

Figure 37. Sedimentation patterns of protein of TMV type strain at 20^{o}C, after being titrated from about pH 5.5 up to the indicated pH value. Protein at 5 mg/ml in 50 mM KCl was titrated to the desired pH and a 1 ml sample was removed. Molar $CaCl_2$ containing 50 mM KCl was added to the remainder to achieve a Ca^{2+} concentration of 27 mM, the pH was restored with alkaline titrant, and a 1 ml sample was again removed. After the addition of 100 μl of I=0.1 cacodylate buffer in order to stabilise the pH, both the sample devoid of Ca^{2+} (lower pattern of each photograph) and the sample containing Ca^{2+} (upper pattern of each photograph) were examined in the analytical ultracentrifuge within 2 h of sampling.

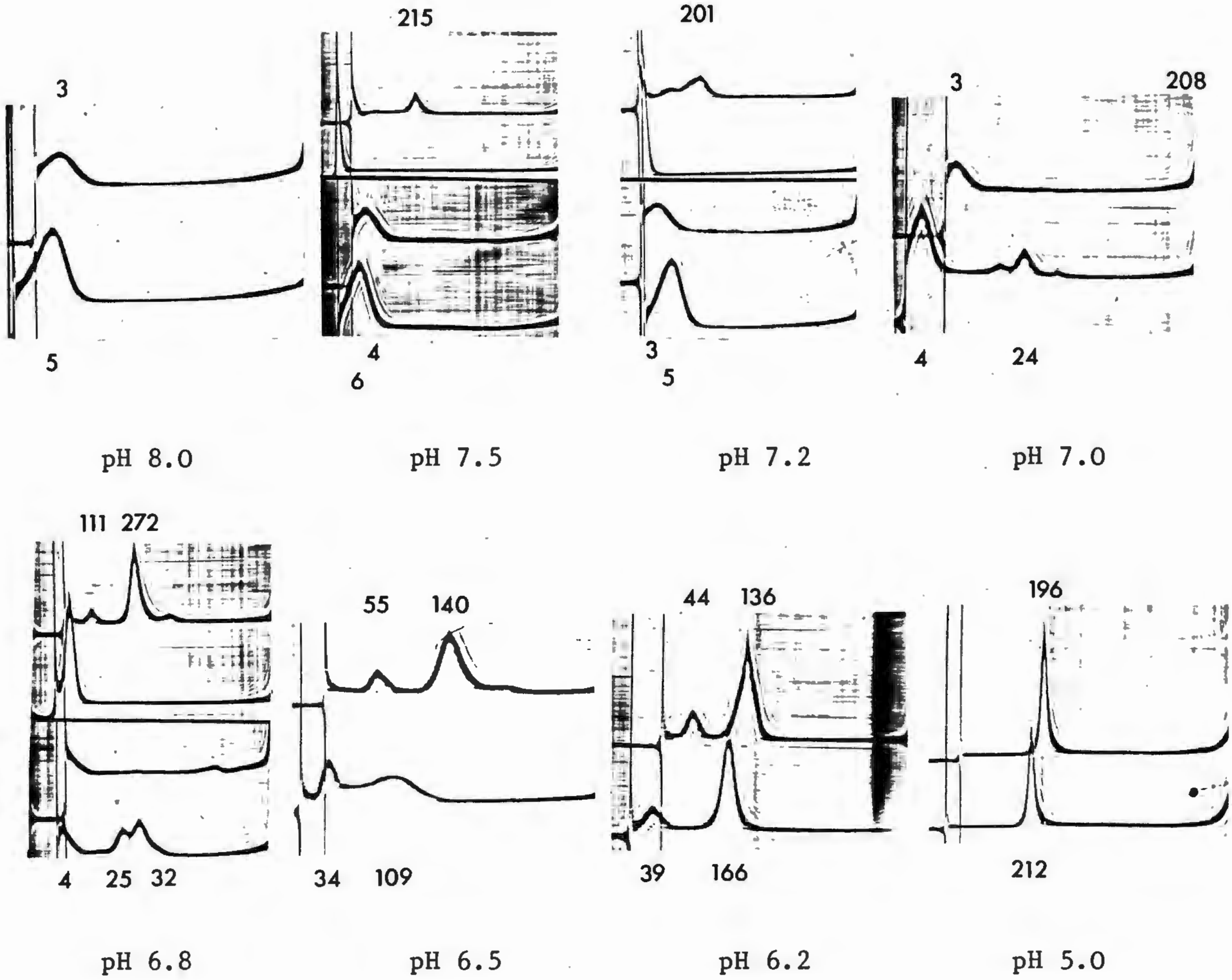


Figure 38. Sedimentation patterns of protein of Y-TAMV at 20°C, after having been dialysed from about pH 5.5 to the indicated pH values. Experimental details are identical to those of Fig. 35. At pH 7.5, 7.2 and 6.8 the upper photograph was taken early during the run to illustrate the fast sedimenting species, while the lower photograph was taken later during the run after the rotational speed of the rotor had been increased.

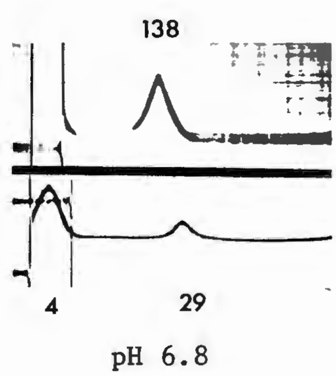


Figure 39. Sedimentation patterns of protein of Y-TAMV at 20°C, after having been titrated from about pH 5.5 up to pH 6.8. Experimental details are identical to those of Fig. 37.

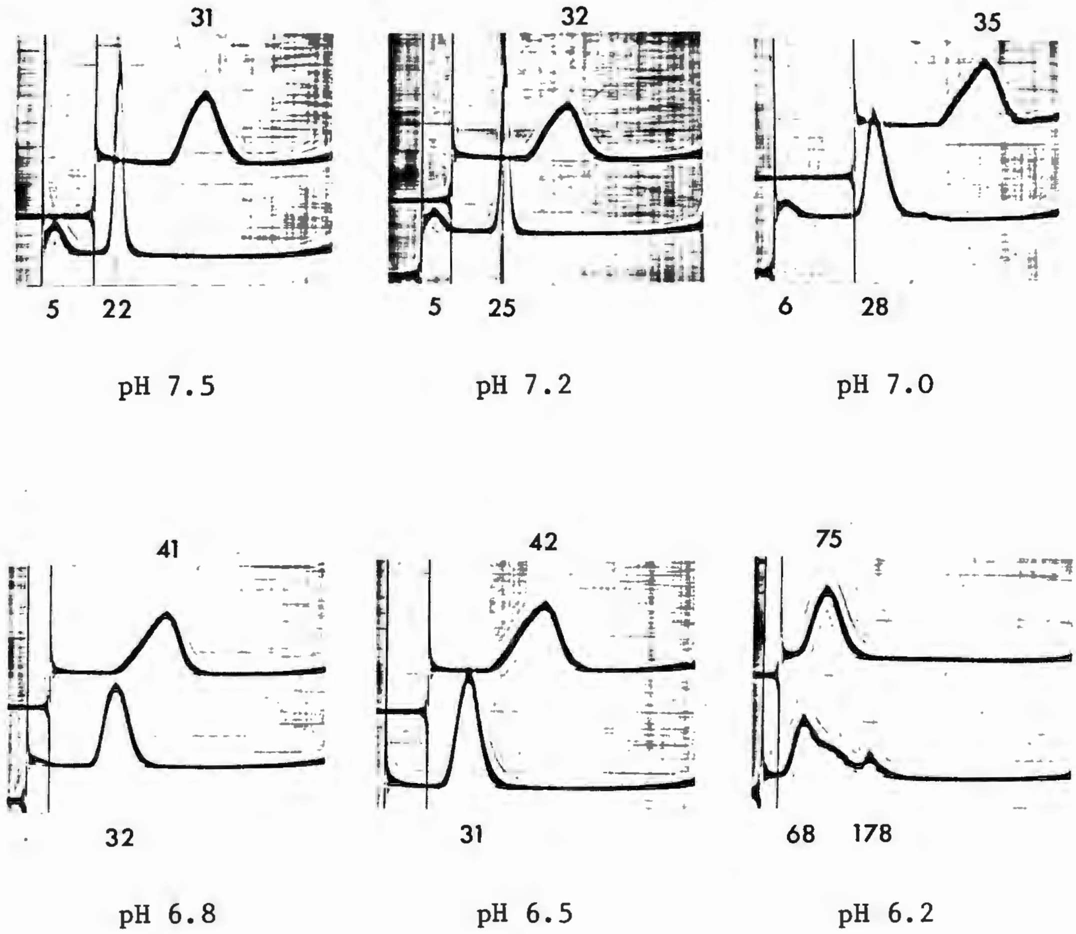


Figure 40. Sedimentation patterns of protein of the cowpea strain of TMV at 20°C, after having been dialysed from about pH 5.5 up to the indicated pH values. Experimental details are identical to those of Fig. 35.

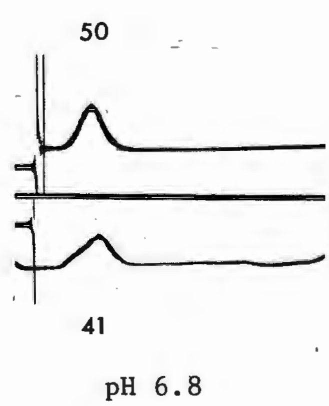


Figure 41. Sedimentation patterns of protein of the cowpea strain of TMV at 20°C, after having been titrated up to pH 6.8. Experimental details are identical to those of Fig. 37.

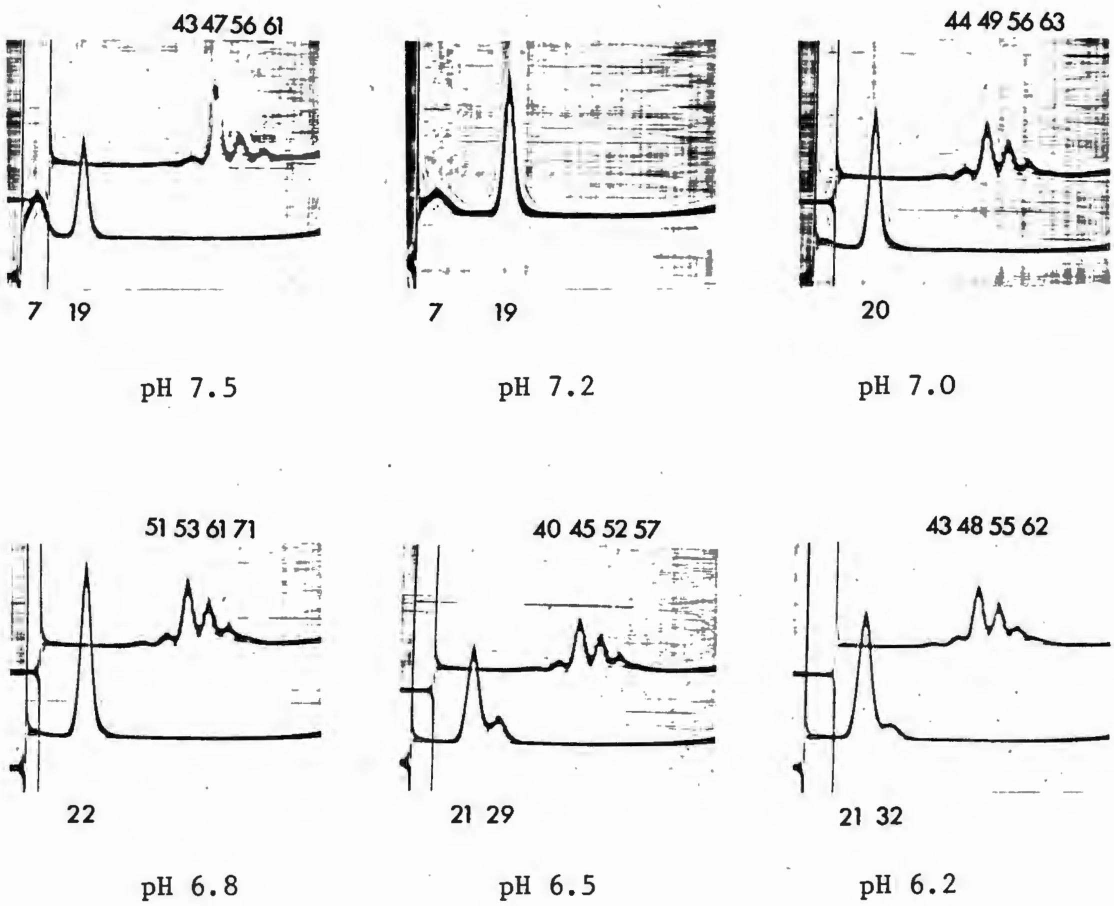


Figure 42. Sedimentation patterns of protein of the U2 strain of TMV at 20°C, after having been dialysed from about pH 5.5 up to the indicated pH values. Experimental details are identical to those of Fig. 35. At pH 7.2 U2P was examined only in the absence of Ca^{2+} ions.

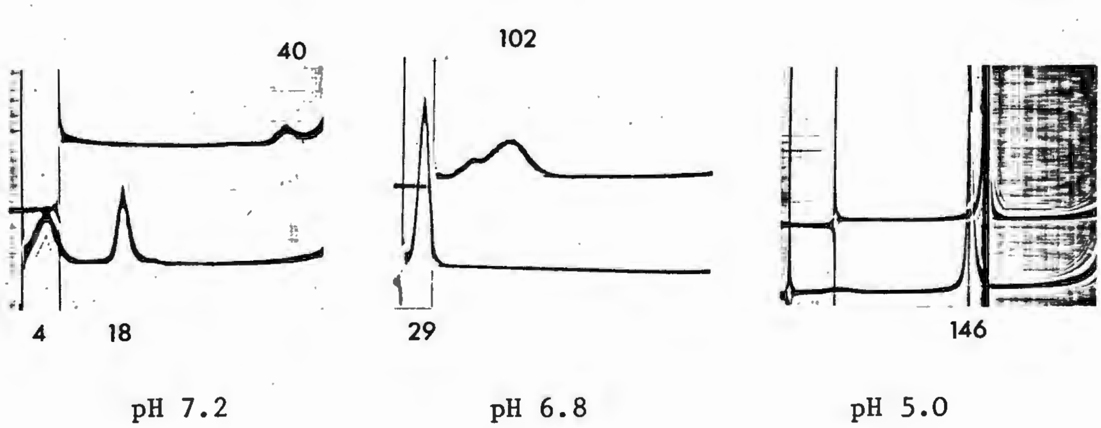


Figure 43. Sedimentation patterns of protein of the U2 strain of TMV at 20°C, after having been titrated to the indicated pH values. Experimental details are identical to those of Fig. 37.

4. Discussion

Working at a free calcium concentration of about 10^{-2} M and TMVP concentrations of 0.8-1.0 mM, McMichael & Lauffer (1975) reported that the approximately two calcium ions per subunit which were bound by TMVP below pH 6.0 were released on raising the pH to 6.5. Shalaby _et al_. (1968) had previously reported that at TMVP concentrations of 0.7-0.9 mM and a calcium concentration of about 10^{-1} M, TMVP bound two calcium ions per protein subunit only at pH 3.2, but that no binding occurred between pH 4.4 and pH 9.4. The results of the present study indicate that at a TMVP concentration of 0.3-0.6 mM and a calcium concentration of about 10^{-3} M very little calcium is bound by TMVP between pH 5.5 and pH 7.8. Although the calcium concentrations used in the above three instances differ considerably, the results of the present study are compatible with those of Shalaby _et al_. (1968). However, neither of these two studies followed the exact sequence of operations used by McMichael & Lauffer (1975). It is thus conceivable that a form of TMVP able to bind calcium could exist at 20^{o}C in a low ionic strength medium of pH 5.0-5.5. However, a comparison of the results in Table 14 with those in Fig. 4 indicates that above pH 6.5, where at pCa values of 2.7-3.0 maximum binding of Ca^{2+} ions by TMV occurs, TMVP binds less than 0.1 Ca^{2+} ions per subunit. TMV protein, even in the long helix, binds substantially less calcium than the virion, indicating that the presence of the RNA is essential for the binding of cations by the intact virus particle. This suggests that the majority (if not all) of the divalent cation-binding sites on TMV vulgare are situated in the "carboxyl cage" of Stubbs _et al_. (1977).

A comparison of the titration curve of divalent cation-free TMVP with that of TMV indicates that TMVP has lost a group which in TMV titrates above pH 7. This observation suggests that TMVP has lost the pK_H 8.3 group of the virion, although interpretation of the TMVP titration curve is complicated by the fact that TMVP changes its aggregation state near pH 7. However, the irreversibly-aggregated "stacked-disc" form of TMVP also appears to lack the group titrating above pH 7 (Butler et al., 1972). These authors ascribed the absence of this group to the packing perturbation shown by the protein subunits of the stacked-disc rods and maintained that a helical aggregate would have titrated similarly to the virus. It seems, however, that the presence of RNA is essential for the formation of the pK_H 8.3 group, especially in the light of the results of Stubbs et al. (1977) on the RNA binding site, and the report of Champness et al. (1976) that the protein disc of TMV has a disordered configuration at radii below 40Å. Durham et al. (1977b) have presented evidence suggesting that the disc form of TMVP contains only one group (corresponding to site 2 of TMV) with a raised pK_H, that the single helix contains two such groups (corresponding to site 1 and site 2), and that the pK_H of site 3 is only raised after the incorporation of RNA into the structure to form the virion.

The titration hysteresis shown by TMVP at pH 6.2 is due to a lag in the depolymerisation of metastable protein helices as the pH is raised above pH 5.5 (Scheele & Schuster, 1975). A second hysteresis occurring near pH 6.9 can be associated with discs breaking down to smaller aggregates (Durham et al., 1977b). The reverse titration (i.e. high pH to low pH) shows no such lags and represents the equilibrium branch of the titration curve (Scheele & Schuster, 1975). The present study indicates that the addition of Ca^{2+} ions to a 27 mM concentration (but not to 1.5 mM) significantly reduces the titration hysteresis, such that both the forward and reverse curves tend to follow the equilibrium branch. The presence of 27 mM Ca^{2+} ions thus accelerates protonation equilibria, although not necessarily the association equilibria, of TMVP.

The graph of protons displaced from TMVP by 27 mM calcium (Fig. 31B) indicates that a maximum of less than 0.5 protons per subunit is displaced from TMVP at pH 6.2. At this pH both stacks of discs and helices are present, postulated to contain respectively one and two raised-pK_H groups per subunit (Durham et al., 1977b).

Protons should theoretically be displaceable from both of these groups by Ca^{2+} ions. The low level of proton displacement indicates either that TMVP does not contain the postulated raised pK_H groups (i.e. calcium is binding to groups which are unprotonated), or that the sites in TMVP have a reduced affinity for Ca^{2+} ions relative to those in the virion. The titration behaviour of TMVP (Butler et al., 1972; Durham et al., 1977b) indicates that the former possibility is unlikely. The titration curves of Butler & Durham (1972) indicate that abnormally titrating groups can be demonstrated on the protein helix, but are absent on the stacked-disc rods.

It thus appears that two of the groups that bind Ca^{2+} ions on the virion are also present on the protein helix, but are altered (perhaps by the absence of RNA) to have a markedly reduced affinity for calcium. The equilibrium dialysis results support this interpretation. Nevertheless, calcium ions reduce the titration hysteresis of TMVP and stabilise the aggregated forms (helix and disc) of the protein. At pH 7.0-7.2, Ca^{2+} ions do not affect the distribution of sedimenting species of TMVP (Fig. 35). At pH 7.6, where normally only an 8 S species is present, Ca^{2+} ions induce the formation of higher aggregates leading to a 21 S species (disc) and a 29-31 S species (double disc). This aggregation is not accompanied by proton release or uptake (Fig. 31B), and is probably due to the calcium ions reducing the electrostatic repulsion between subunits. The slight calcium-induced proton consumption by TMVP at pH 6.8-7.0 (Fig. 31B) is probably due to the formation of discs resulting in the pK_H of one group being raised to about 6.9 (Durham et al., 1977b).

Between pH 6.8 and pH 6.2 calcium ions induce the formation of larger aggregates (~35 S, ~60 S, ~76 S) but not of long protein helices (>100 S). The equilibrium distribution of Ca^{2+}-free TMVP in this range is discs (20-30 S) giving way to small helical aggregates (50-70 S) as the pH is lowered (Scheele & Schuster, 1975; Durham et al., 1977b). Calcium ions thus favour a shift of the equilibrium towards stacks of discs leading to short helices, possibly by reducing the electrostatic repulsion that keeps the two layers of the disc apart near the axis and prevents the formation of protein helices. If, however, calcium ions are added at pH 6.2 during an upward (i.e. non-equilibrium) titration of TMVP, the metastable helices (>100 S) are induced to undergo limited depolymerisation to stable smaller aggregates with an accompanying release of protons (Fig. 31B). The pH of maximum proton release coincides with the pH of maximum hysteresis (curve d, Fig. 31A), indicating that calcium ions are either converting metastable helices with two raised-pK_H groups per subunit to stable stacks of discs with only one raised-pK_H group per subunit, or are binding to helices (thereby displacing protons) and simultaneously causing depolymerisation to a stable equilibrium situation, possibly to short helices. The mechanism of this calcium-induced conversion is obscure and its elucidation awaits a clarification of not only the factors responsible for the metastability of the helix at pH 6.2, but also of the exact nature of the equilibrium aggregates existing at that pH and of the requirements for true stable helices.

The stabilisation of polymerised protein by Ca^{2+} ions was postulated by McMichael & Lauffer (1975) to involve two forms of TMV helix existing at low pH. The descending branch of the titration curve of TMVP (curve a,

Fig. 31A) contains a step at pH 5.9, which Durham et al. (1977b) ascribed to a cooperative conformational change leading to true single helices. This step is absent if TMVP is titrated in the presence of 27 mM Ca^{2+} (curve c, Fig. 31A). Although in the present study minimal binding of Ca^{2+} ions to TMVP was observed at pH values below the step (Table 14), these determinations were done at a calcium concentration of 2 mM which is a five-fold lower calcium concentration that that used by McMichael & Lauffer (1975).

The distribution of sedimenting species of TMVP observed at pH 6.5 in the presence of Ca^{2+} ions was not the same as that reported by McMichael & Lauffer (1975). At pH 6.5 and 20^{o}C they observed a single sedimenting peak of 248 S in the presence of calcium, whereas only a 60 S peak was observed in the present study (Fig. 35). On cooling their TMVP solution to 7^{o}C, they observed only a 3 S form in both the calcium-containing and the control solutions, while a 25-27 S peak can be seen in Fig. 36. The TMVP solutions of McMichael & Lauffer (1975) were probably not at association equilibrium, however.

The ability of Ca^{2+} ions to induce depolymerised protein to form discs is clearly seen if TMVP is dialysed against calcium at 4^{o}C (Fig. 36), or if calcium is added to TMVP during a titration (Fig. 37). The latter situation does not represent association equilibrium; although the polymerisation induced by the addition of Ca^{2+} at pH 7.2 appears to equilibrate rapidly (cf. the pH 7.2 frames of Figs 35 and 37), the addition of calcium at pH 6.8 during a titration causes a polymerisation overshoot (pH 6.8 frame of Fig. 37) similar to that caused by the rapid addition of acid to TMVP at that pH (Scheele & Schuster, 1975).

The titration behaviour of YP was very similar to that of TMVP; in particular, the patterns of proton displacement by calcium from the two strains was almost identical (cf. Figs 31B and 32B). The aggregation behaviour of divalent cation-free YP as a function of pH (Fig. 38, lower patterns of each frame) also resembles that of TMVP, except that in the case of YP long helices (>100 S) were evident at pH $\leq$ 6.5. There was no evidence of the ~100 S component observed at pH 7.0 by Rentschler (1967), while at pH ~6 the protein helices (>100 S) were found to be the major component present and not the ~20 S species reported by her.

A feature of the YP that has been dialysed against calcium was the presence of a species sedimenting at >200 S at pH values $\leq$7.5. Although discs (20-40 S) were present in preparations of divalent cation-free YP (Fig. 38, lower patterns), there was no evidence of discs in YP preparations that had been dialysed against calcium. Calcium ions were thus favouring the formation of large aggregates, presumably helical rods. Prior to dialysis and ultracentrifugal analysis, YP preparations (and, in fact, the proteins of the other three strains as well) had originally been in the helical form at pH ~5.5 following the EDTA/KCl treatment to remove divalent cations. Subsequent dialysis of this protein against a high pH buffer containing calcium thus resulted in stabilisation of the helices at pH values where, in the absence of calcium ions, the normal depolymerisation pattern was followed. Also, a 46-55 S species was present in the preparations containing calcium at pH 6.2 and 6.5, but this species depolymerised at higher pH values. In the presence of calcium, helices were stabilised up to pH 7.5, but at pH 8.0 were completely depolymerised to A-protein.

The calcium-induced consumption of protons by YP at pH 6.8 (Fig. 32B).

can be explained, as for TMVP, by the conversion of A-protein and discs to helices (Fig. 39).

Calcium ions displace significantly more protons from U2P (Fig. 33B) than from either TMVP or from YP. As a maximum of nearly two protons per subunit was displaced by 27 mM Ca^{2+} at pH 6.0, U2P must possess two calcium binding sites per subunit, each having a fairly high affinity for the cation. TMVP and YP possess at most one site per subunit having only a weak affinity for calcium. The rapid decrease above pH 6.3 in protons displaced from U2P by calcium, which parallels the drop in titration hysteresis (curve d, Fig. 33A), suggests that the ability to bind calcium ions is lost upon depolymerisation of the protein. Although (for reasons not understood) this depolymerisation is not reflected in the lower patterns of the frames of Fig. 42, Rentschler (1967) working at I=0.1 reported that U2P existed only as an aggregate of >100 S at pH 6.0, while only another of <10 S (A-protein) was discernible at pH 7.0. The titration hysteresis observed with U2P indicates that depolymerisation must have occurred near pH 6.5 in the same way as with TMVP.

Calcium binding by U2P thus appears to be a cooperative process requiring protein polymerisation. The binding sites could be composed either of juxtaposed ligands originating from two separate but adjacent subunits, or of intrasubunit structures formed only when the polypeptide is forced by aggregation to assume a particular orientation. The formation of these two sites obviously does not require the presence of RNA. In the case of TMVP (and thus probably U2P as well) the incorporation of the RNA affects the orientation of the polypeptide in its immediate vicinity (Champness _et al._, 1976; Stubbs _et al._, 1977). As these two sites are also present in the U2 virion, one can thus conclude that the two metal binding sites on U2P lie at radii greater than 40 Å in the polymerised protein.

The sedimentation behaviour observed at various pH values with divalent cation-free U2P (Fig. 42, lower patterns of each frame) was markedly different from that observed by Rentschler (1967). No indication was obtained in the present study of the >100 S species which she observed near pH 6.0, and conversely she did not observe the 19-20 S species obtained in the present study from pH 7.0 upwards. U2P was in the helical form prior to dialysis, and titration to pH 5.0 or pH 6.8 in the presence of Ca^{2+} ions, resulted in the formation of species sedimenting at >100 S. The reason for the absence of large aggregates after prolonged dialysis of U2P near pH 6.0 is not understood.

Addition of calcium to U2P shifted the equilibrium towards larger aggregates. The fact that the distribution of protein polymers observed after dialysis against calcium-containing buffers was nearly constant from pH 6.2 to pH 7.5 (Fig. 42, upper pattern of each frame), suggests that the presence of Ca^{2+} ions at low pH could have "locked" the protein into a series of short stacked-disc or helical structures which subsequently did not dissociate even at pH 7.5. A similar effect was observed if Ca^{2+} ions were added at pH 7.2 during a titration (Fig. 43), but not at pH 6.8. The rapid addition of calcium at this latter pH value could possibly have caused a polymerisation overshoot, which would then have equilibrated with time to resemble the position observed after dialysis at that pH (Fig. 42). If, as discussed earlier, Ca^{2+} ions are binding to a site on U2P involving adjacent subunits, these cations could conceivably serve to link the subunits and stabilise them in the polymerised form at higher pH values.

The titration curve of divalent cation-free CP (curve a, Fig. 34A) was unique for the absence of hysteresis. Surprisingly, the curve was similar to the upward, non-equilibrium branch of TMVP's titration curve (see curve a, Fig. 31A). The displacement of protons from CP (Fig. 34B) suggested that this protein also contains two binding sites per subunit, having a weaker affinity for Ca^{2+} ions than those on U2P. The rapid decrease above pH 6.8 in protons displaced by calcium suggests that binding of the cation is a cooperative process in the case of CP as well as U2P.

The sedimentation behaviour of divalent cation-free CP (lower patterns of each frame, Fig. 40) was similar to that of TMVP, with large >150 S aggregates being present below pH 6.5, 5 S aggregates being present above pH 7.0, and single or double discs being present above pH 6.5. The sedimenting species observed after dialysis did not suggest a depolymerisation event at pH 6.9 as was suggested by Fig. 34B; however, the two experimental systems were observing equilibrium and non-equilibrium situations respectively. As with U2P it appeared that the presence of calcium ions was stabilising a short stacked-disc or helical structure of CP formed below pH 6.0. These structures then resisted depolymerisation at higher pH values; no protein species of less than 31 S was observed in the presence of calcium even at pH 7.5.

A general picture of calcium binding to the proteins of the four TMV strains thus emerges. TMVP and YP respond similarly to Ca^{2+} ions in that very little binding of calcium occurs. However, the larger aggregates of both these proteins are stabilised at pH values where normally dissociation would have occurred. This was particularly evident with YP where in the presence of Ca^{2+}, a species sedimenting at >200 S was observed at a pH as high as 7.5. This stabilisation is possibly due to calcium ions in the medium reducing the electrostatic repulsion between subunits. In the case of U2P and CP, Ca^{2+} ions stabilise short stacked-disc or helical aggregates such that they do not depolymerise even at elevated pH values. As the calcium ions displace almost two protons per subunit from these proteins, this stabilisation is apparently a result of calcium ions being bound to the protein, possibly to a site involving the cooperation of two adjacent subunits. In both cases, the ability of the protein to bind calcium is apparently lost as the protein depolymerises. A possible alternative explanation is that calcium is able to bind to these two proteins when depolymerised (i.e. at pH values above 7), but that depolymerisation results in the lowering of the pK_H of the site such that above pH 7 the site is deprotonated (i.e. contains no displaceable protons). However, if depolymerisation affects the structure of the site sufficiently to cause a lowering of the pK_H, one would expect the calcium binding affinity to be affected as well. A measurement of the binding of calcium to these two proteins as a function of pH, using equilibrium dialysis, would point to the correct explanation. Calcium ions probably have an electrostatic effect on the polymerisation of these two proteins as well.

Calcium ions are known to influence the polymerisation equilibria of certain other proteins that normally undergo association. Neurophysin exists as a monomer of about 11000 daltons at low concentration and as a multimer of about 35000 daltons at higher concentrations, while in the presence of 10^{-5} M calcium a single, concentration-independent species of 21000 daltons is obtained (Burford et al., 1971). A 17 S species of lobster haemoglobin dimerises rapidly and reversibly to a 25 S species in the presence of 5-14 mM Ca^{2+} (Morimoto & Kegeles, 1971). A 7.2 S tetramer of mushroom tyrosinase dissociates to 4.4 S and 6.1 S forms after dialysis against 1 mM EDTA. This effect is reversed by 1 mM Ca^{2+} which ions can subsequently be removed by dialysis leaving the tetramer intact (Jolley et al., 1969). The 5.8 S monomer of tubulin is induced to aggregate reversibly by the addition of Mg^{2+} or Ca^{2+}, a 30 S aggregate predominating at 10 mM calcium concentration (Weisenberg & Timasheff, 1970). None of the above authors commented, however, on the mechanism whereby divalent cations could be inducing polymerisation of the proteins. Jolley et al. (1969) reported that calcium ions were not bound tightly by the tyrosinase, and that Zn^{2+}, Cu^{2+} and Pb^{2+} ions could not reverse the EDTA-induced dissociation as could Ca^{2+}. They speculated that juxtaposed tyrosinase subunits were positioned by loose binding of calcium ions which then enabled other forces to take over.

E. Titration of spherical viruses

The titration behaviour of a number of spherical viruses, both in the absence and the presence of divalent cations, was also investigated in the present study. This was undertaken to establish whether the phenomenon of cation binding leading to an alteration in titration behaviour could be demonstrated for viruses other than TMV. The viruses chosen were turnip yellow mosaic virus (TYMV) which has been reported to bind cations (Johnson, 1964), turnip crinkle virus (TCV) which undergoes a marked destabilisation as the pH is raised above 7.5 (Durham, 1971) and bromegrass mosaic virus (BMV) which swells and becomes susceptible to enzymes above pH 7 when divalent cations are absent (Incardona *et al.*, 1973).

1. Turnip yellow mosaic virus

TYMV that had been freed of divalent cations was titrated in 50 mM KCl, at concentrations ranging from 2 to 5 mg/ml. The titration curve was reproducible (curve a, Fig. 44), and a total of 12.2 protons per subunit titrated between pH 4.5 and pH 8.5. During a titration, the pH of the virus solution equilibrated slowly, the readings tending to rise rapidly after each addition of alkali and to drop slowly to the equilibrium position. The titration curves obtained in the presence of 1.5 mM Ca^{2+} (curve b, Fig. 44) and 27 mM Ca^{2+} (curve c, Fig. 44) were both very similar to that obtained in the absence of calcium, and at no pH was more than half a proton per subunit displaceable by calcium.

2. Turnip crinkle virus

Divalent cation-free TCV was titrated in 50 mM KCl at concentrations ranging from 3 to 6 mg/ml. A total of about 15.5 protons per subunit titrated between pH 4.5 and pH 8.5 (curve a, Fig. 45), and the curve was characterised by a hysteresis loop centered on pH 7.7 (curve c, Fig. 46). The hysteresis loop had an amplitude at pH 7.6 of 0.97 protons per subunit, but in this region the pH meter readings were always changing in a direction towards pH 7.7, suggesting that a titration curve established infinitely slowly would have shown a sharp step at that pH.

Titration of TCV in the presence of 1.5 mM Ca^{2+} (curve b, Fig. 45) and 27 mM Ca^{2+} (curve c, Fig. 45) produced curves without the hysteresis loop. Calcium at 1.5 mM concentrations displaced a maximum of one proton per subunit at pH 5.6 (curve a, Fig. 46), while 27 mM Ca^{2+} displaced a maximum of 2.5 protons per subunit at pH 5.3 (curve b, Fig. 46). Curves (a) and (b) of Fig. 46 are remarkably similar to the analogous curves for TMV type strain (curves a and b, Fig. 6B). The protons involved in TCV hysteresis are displaceable by Ca^{2+} (Fig. 46).

3. Bromegrass mosaic virus

BMV, freed of divalent cations, was titrated in 50 mM KCl at concentrations ranging from 4 to 6 mg/ml. A total of about 8.3 protons per subunit titrated between pH 4.5 and 8.5 (curve a, Fig. 47), and the titration curve contained a prominent hysteresis loop. The hysteresis amounted to a maximum of 1.6 protons per subunit at pH 6.4 (curve c, Fig. 48). The titration curve obtained in the presence of 1.5 mM Ca^{2+} (curve b, Fig. 47) had a reduced hysteresis loop, amounting to a maximum of 1.1 protons per subunit at pH 6.5 (curve d, Fig. 48). In addition, 1.5 mM Ca^{2+} displaced a maximum of 0.8 protons per subunit at pH 5.2 (curve a, Fig. 48).

The addition of 27 mM Ca^{2+} substantially closed the hysteresis loop (curve c, Fig. 47; curve e, Fig. 48). Slight hysteresis was still present, centered on pH 5.7. A maximum of 2.5 protons per subunit were displaced at pH 5.1 by 27 mM Ca^{2+} (curve b, Fig. 48). The hysteresis most probably corresponded to the two titrating groups, the protons of which are displaceable by Ca^{2+} (Fig. 48). Titration curves obtained in the presence of magnesium indicated that its effect was very similar to that of calcium.

The fact that the hysteresis loop did not close until the virus solutions were titrated down to pH 4.5 (see Fig. 47) appeared to be related to another observation. Virus that had been titrated to above the start of hysteresis at pH 6.5 became cloudy if titrated back below pH 5.4. However, addition of 27 mM Ca^{2+} at pH 5.4 clarified the turbid solution.

4. Discussion

(a) Turnip yellow mosaic virus

Although purified TYMV has been reported to contain substantial amounts of bound metal ions (Johnson, 1964), in the present study no significant displacement of protons by Ca^{2+} ions was detected from TYMV that had been dialysed against EDTA. Kaper (1971) has observed that the RNA of TYMV is linked to the protein by means of groups titrating near neutrality. However, it was only after perturbation of the strong protein-protein bonds of the capsid that groups linking the RNA and protein could be demonstrated, and that various other titratable groups were unmasked. In addition, TYMV retains its polyamines strongly, in contrast to their ready loss from viruses such as BMV (Pfeiffer & Durham, 1977). Johnson (1964) suggested that the cations bound to TYMV resisted removal by EDTA.

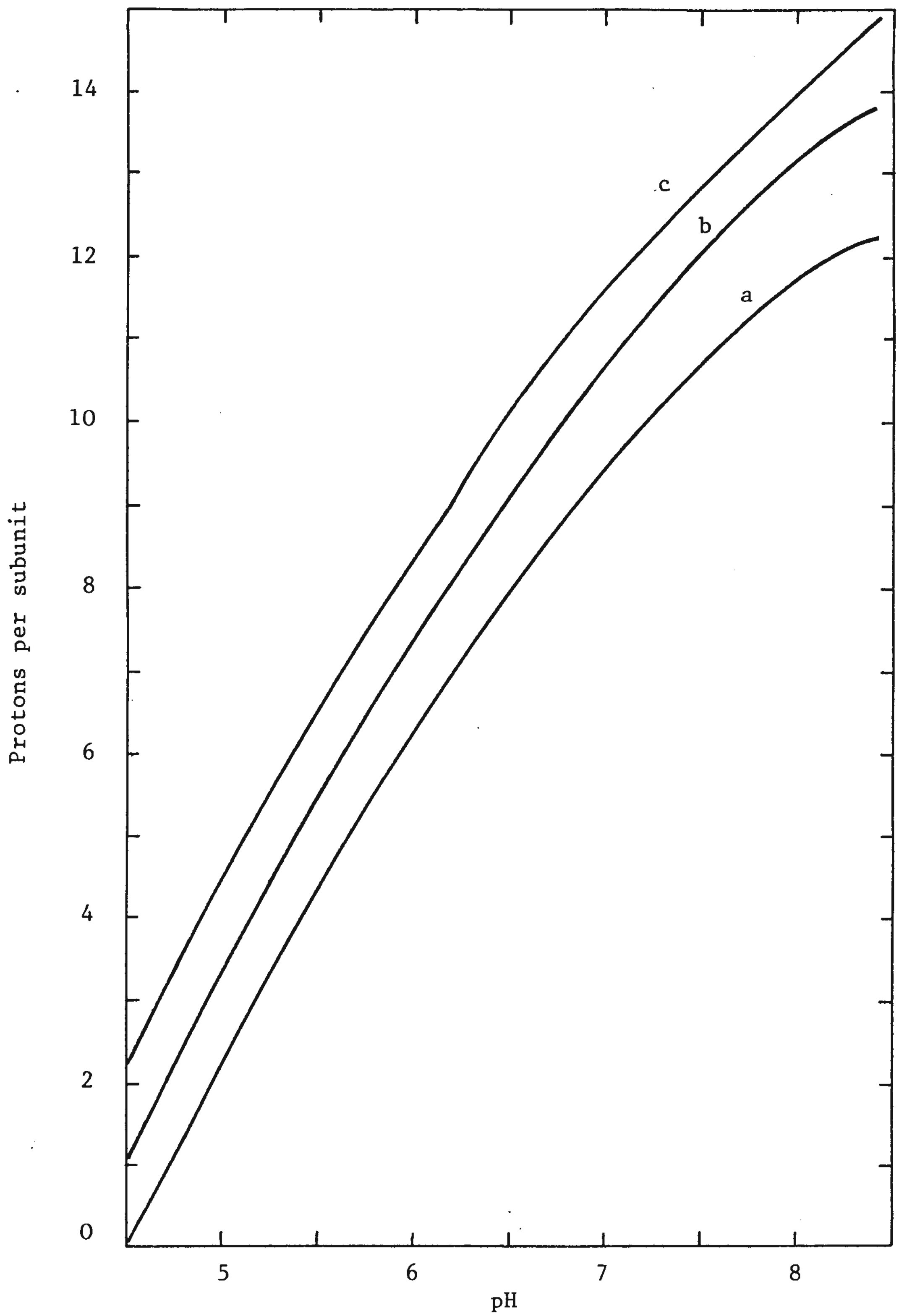


Figure 44. Titration curves of TYMV in the presence of calcium. TYMV was titrated in the absence of divalent cations (a), and in the presence of 1.5 mM Ca^{2+} (b) and 27 mM Ca^{2+} (c). The vertical positioning of the curves is arbitrary. Nowhere between pH 4.5 and 8.5 was more than 0.5 protons displaced per subunit by addition of calcium.

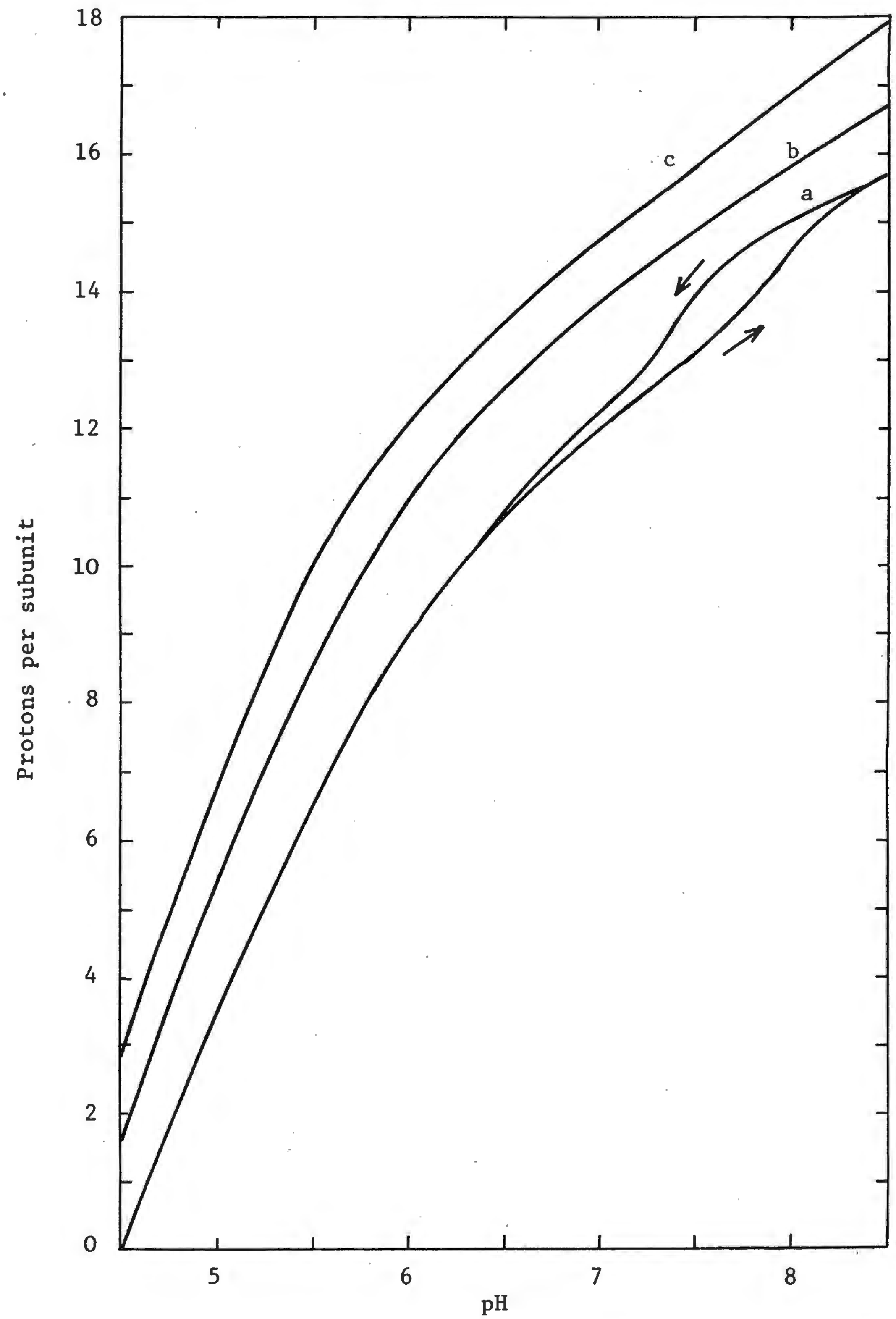


Figure 45. Titration curves of TCV in the presence of calcium. TCV was titrated in the absence of divalent cations (a), and in the presence of 1.5 mM Ca^{2+} (b) and 27 mM Ca^{2+} (c). Arrows indicate forward (alkali titrant) and reverse (acid titrant) titrations. Curves (b) and (c) have been accurately positioned relative to (a), but for clarity both have been shifted upwards by one proton per subunit.

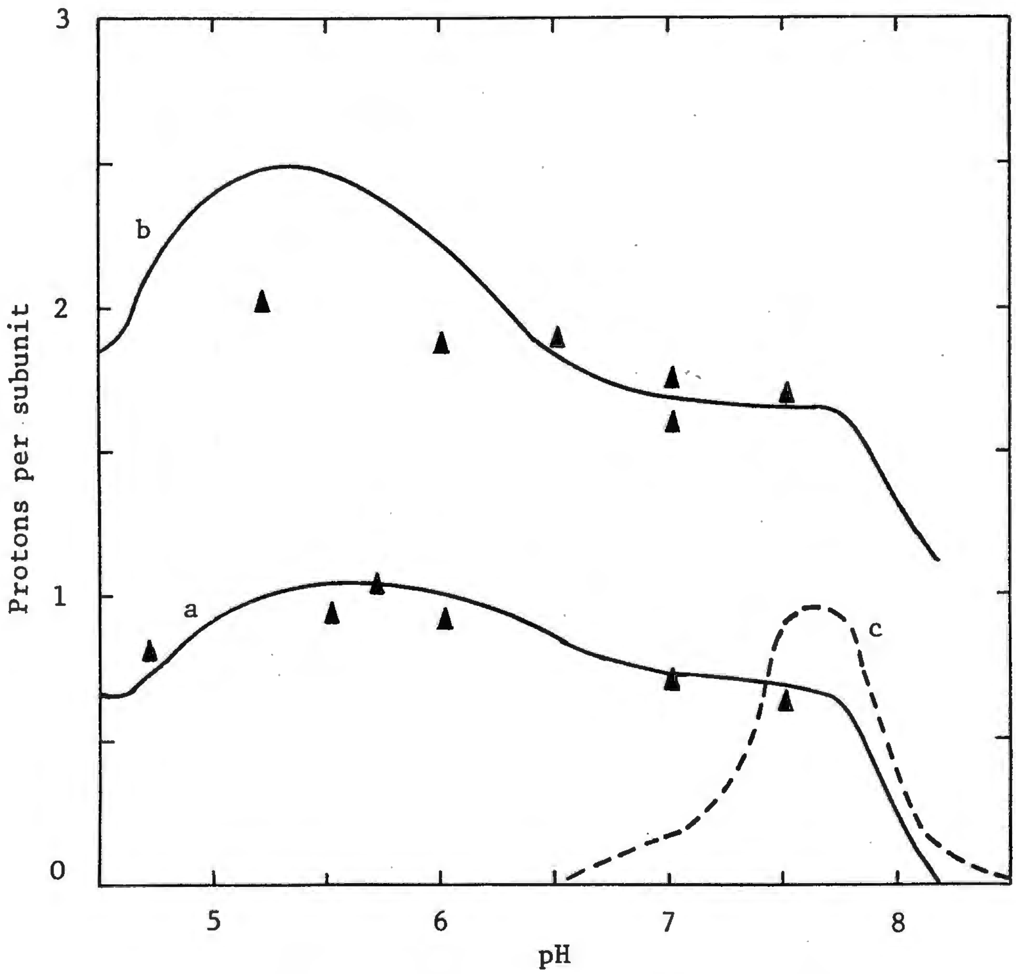


Figure 46. Proton displacement and titration hysteresis curves for TCV. The continuous curves, derived from the data in Fig. 45, illustrate the number of protons displaced from TCV, as a function of pH, by 1.5 mM Ca^{2+} (a) and 27 mM Ca^{2+} (b), and were accurately positioned vertically by means of displacement values (triangles) obtained by addition of Ca^{2+} to aliquots of TCV. The dotted curve (c), derived from curve (a) of Fig. 45, represents the titration hysteresis of divalent cation-free TCV.

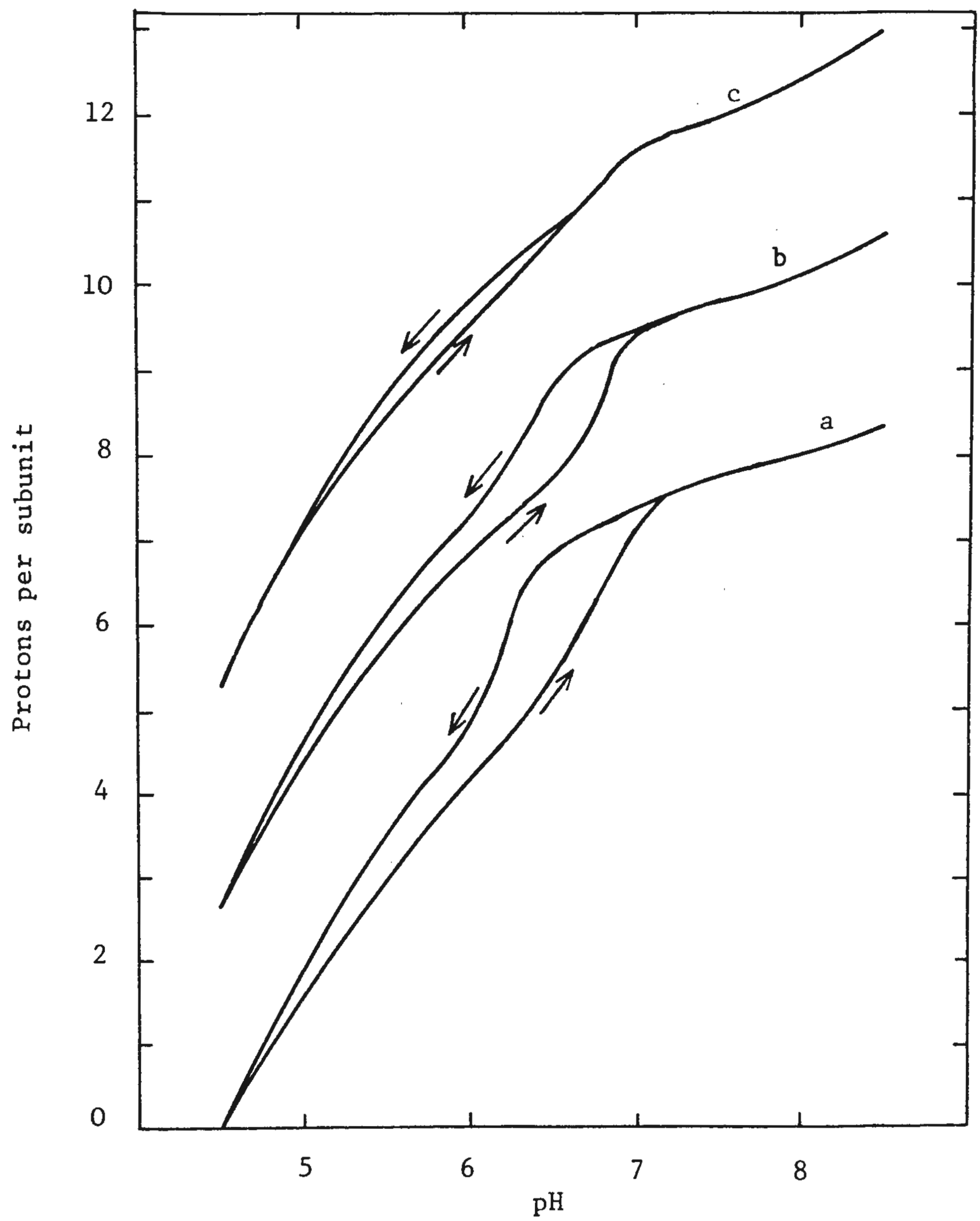


Figure 47. Titration curves of BMV in the presence of calcium. BMV was titrated in the absence of divalent cations, and in the presence of 1.5 mM Ca^{2+} (b) and 27 mM Ca^{2+} (c). Arrows indicate forward (alkali titrant) and reverse (acid titrant) titrations. Curves (b) and (c) have been accurately positioned relative to (a), but for clarity have been shifted upward by two and by four protons per subunit respectively.

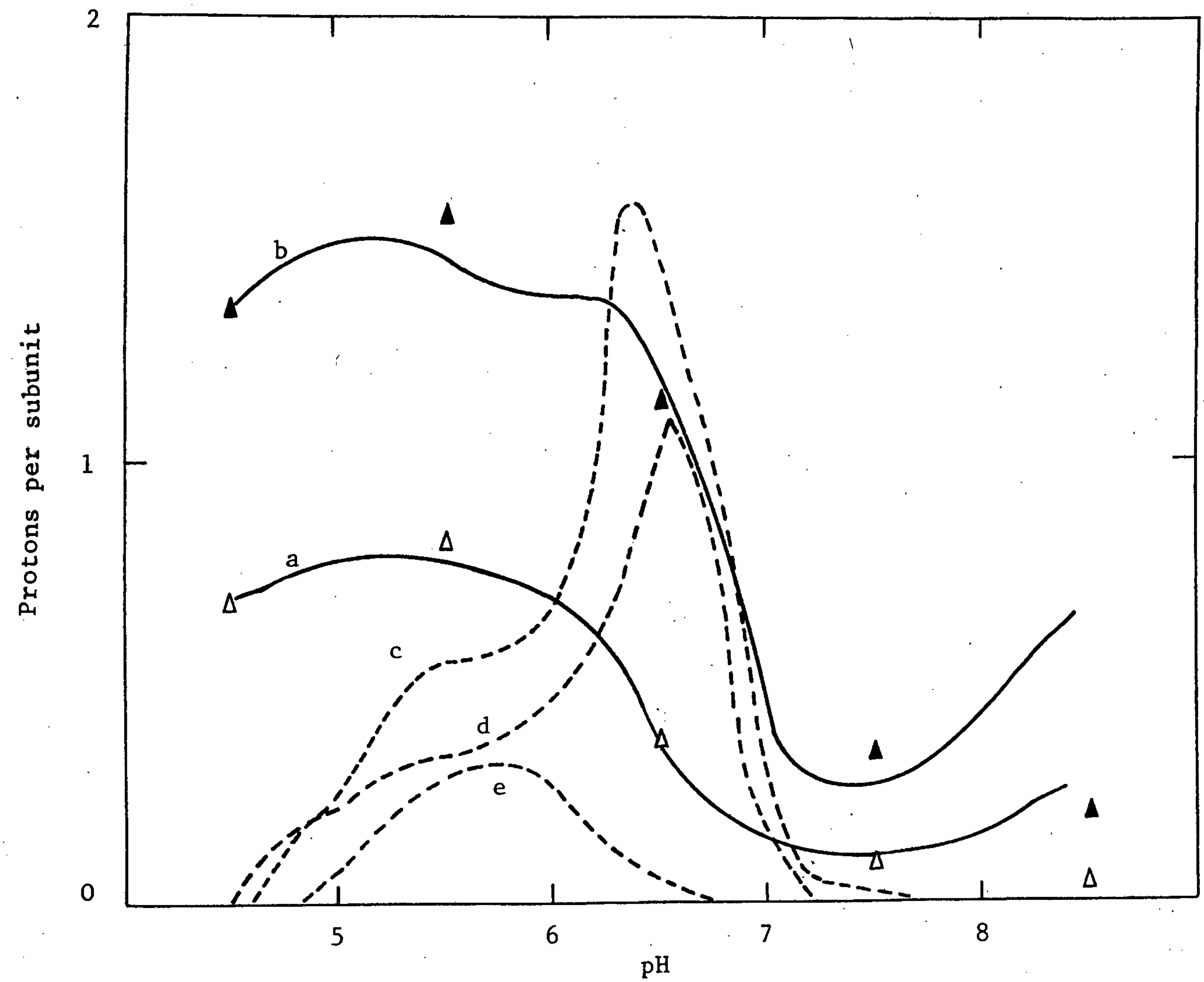


Figure 48. Proton displacement and titration hysteresis curves for BMV. The solid curves, derived from the data of Fig. 47, illustrate the number of protons displaced from BMV, as a function of pH, by 1.5 mM Ca^{2+} (a) and by 27 mM Ca^{2+} (b). The curves were accurately positioned by means of displacement values (triangles) obtained by addition of Ca^{2+} to aliquots of BMV. The dotted curves represent the hysteresis of BMV alone (c), and in the presence of 1.5 mM Ca^{2+} (d) and 27 mM Ca^{2+} (e), and were derived from curves (a), (b) and (c), respectively, of Fig. 47.

An explanation for the above phenomena is that the protein coat of TYMV constitutes a virtually impermeable barrier to small molecules. This phenomenon, which has also been proposed for certain bacteriophages (Ames & Dubin, 1960), would prevent access of EDTA molecules to the cations already bound to TYMV, or would prevent access of Ca^{2+} ions to vacant binding sites in the virion. Capsid perturbation is thus required to demonstrate both cation binding to the virion, as well as the titration of formally masked groups. The slow pH equilibration observed in the present study during titrations also suggests restricted access of titrant ions to the prototropic groups in TYMV.

(b) Turnip crinkle virus

The presence of the hysteresis loop at pH 7.7 in the titration curve of divalent cation-free TCV is interpreted in the present study to mean that TCV undergoes a structural transition between tight and loose forms at that pH. The hysteresis amounted to nearly one proton per subunit at pH 7.7 (curve c, Fig. 46), indicating that this structural transition is controlled by the titration of one or two groups per protein subunit. The groups that can be expected to titrate near neutrality in TCV are the three histidine residues and possibly a terminal amino group (Butler, 1970). However, about 6.4 protons per subunit titrate between pH 6.0 and pH 8.5 (curve a, Fig. 45). This anomalous titration behaviour, together with the ability of Ca^{2+} ions to bind to TCV and displace protons, strongly suggests the presence of abnormally titrating carboxylates in TCV.

It is noteworthy that the protons involved in the titration hysteresis are also the ones that are displaceable by Ca^{2+}. This indicates that divalent cations bind to the same sites as the protons involved in the structural change, thereby prolonging the stability of the tight form. A comparison of proton displacement by Ca^{2+} from TMV with that from TCV indicates a number of similarities: both viruses have cation binding groups with pK_H near 8 controlling virus dissociation, other groups near 6 also showing cation binding, and groups titrating between those regions showing almost no cation binding.

(c) Bromegrass mosaic virus

As BMV undergoes a pH-controlled structural swelling above pH 6.5 (Incardona et al., 1973), the hysteresis loop, which is centered on pH 6.5 in the absence of calcium, presumably corresponds to this alteration in capsid structure. This capsid swelling appears to be controlled by two titrateable groups per subunit, as the amplitude of the hysteresis loop is clearly greater than one proton per subunit (curve c, Fig. 48).

As was observed with TCV, the protons that are involved in the hysteresis are the ones that are displaceable by Ca^{2+} ions (Fig. 48). Calcium ions are thus binding to the same site as the protons involved in the virus structural change. The presence of divalent cations stabilises BMV and prevents the swelling caused by raising the pH above 6.5 (Incardona et al., 1973). The most logical conclusion is thus that the binding of divalent cations to these sites on BMV counteracts the swelling and prolongs the stability of the compact form. Pfeiffer & Durham (1977) have presented evidence in support of these binding sites being situated at the RNA-protein interface, probably in a region of high electronegativity due to the presence of carboxylate and phosphate groups.

These authors also attempt to explain the clarification by calcium ions of the turbidity induced in a divalent cation-free BMV solution by titration to pH 6.5 and back to pH 5.4. They postulated that, after swelling at pH 6.5 in the absence of calcium ions and subsequent reduction of the pH, the swollen virions contract to an incorrect structure with the RNA partially exposed. This negatively charged RNA cross-links the positively charged virus particles. Addition of divalent cations releases the linked particles, presumably by neutralising charges, and causes at least a partial retraction of the RNA strands into the virion.

(d) General

The binding of divalent cations to a number of spherical viruses has been conclusively demonstrated. In addition, this binding has a direct effect on the structure of the virions concerned, as indicated by the marked reduction in titration hysteresis in the presence of the cation. A compact, enzyme-resistant form of both BMV and TCV is stabilised at pH values where particle expansion normally occurs. In each of these two viruses, the functions of particle expansion and cation binding are apparently controlled by the same raised-pK_H carboxylate groups. This phenomenon is thus not peculiar to TMV and its strains. BMV is the best-characterised member of a group of similar viruses, the capsids of which all appear to respond to the presence of divalent cations in a similar fashion to that of BMV (Lane, 1974). A host of other plant, animal and bacterial viruses are known to either be stabilised by, or to respond structurally to, the presence of divalent cations (Durham *et al.*, 1977a). This phenomenon must thus be regarded as being widespread among viruses, and undoubtedly represents a hitherto disregarded element influencing virus stability.

CHAPTER FIVE

GENERAL CONCLUSIONS

The original postulate of Durham & Butler (1975), that the dissociation of calcium ions from virus particles *in vivo* triggers RNA release, has been shown in the present study to be an oversimplification. Although the *in vitro* studies suggest that a low calcium concentration is a necessary condition for TMV disassembly, various other factors are apparently required for RNA and protein to actually separate *in vivo*; Durham (see Durham *et al.*, 1977a) has argued that this could be a membrane. Disassembly of TMV *in vitro* requires, in addition to the absence of divalent cations, either the presence of bentonite below pH 8, or the elevation of the pH of a bentonite-free solution to above pH 8. It would be fruitful in this context to elucidate the precise action of the bentonite, which according to Brakke (1971) does not adsorb TMV protein subunits.

The four strains of TMV examined in this study bind measurable amounts of calcium. This binding occurs with a sufficiently strong affinity in each case to result in the binding of calcium and the stabilisation of the virion in most extracellular environments. Conversely, in the low calcium concentration of the cytoplasm, calcium ions would dissociate from TMV vulgare; binding constants were not determined for the U2, Y-TAMV and cowpea strains, but these appeared to have higher affinities for calcium than did vulgare. These strains all had a significantly higher affinity for calcium than for magnesium. Thus, although dissociation of calcium ions was not sufficient to cause virus disassembly, the affinity of calcium binding was of the correct order of magnitude for the dissociation of bound calcium ions to occur *in vivo* and to have a biologically significant function. This dissociation could possibly be one of a series of essential prerequisites for virus disassembly in vivo. Alternatively, as postulated by Durham (manuscript submitted for publication), this dissociation could provide the energy for both penetration of the virion through the cell membrane and for subsequent disassembly events.

Although the polymerisation of TMVP still appears to be controlled by two anomalously-titrating groups per subunit, TMV contains three anomalously-titrating groups per subunit. These three groups are probably situated in the "carboxyl cage" of Stubbs et al. (1977), the one group peculiar to the virus possibly involving a protein carboxylate and an RNA phosphate group. All three groups in the virion bind divalent cations, although of the three, the one found only on the virion has the strongest affinity for divalent cations. As this group has a pK_H of 8.3, it would appear to be the one also controlling the in vitro alkaline dissociation of TMV. The titration behaviour, metal ion binding properties, and locality in the virus structure of the pK_H 8.3 group thus collectively assign to it a crucial role in the stabilisation of TMV.

The fact that the proteins of the four TMV strains, particularly of vulgare and Y-TAMV have significantly lower affinities for divalent cations indicates that the binding of these cations is a function of the quaternary structure of the virion. Some residual affinity for metal ions is retained by polymerised U2 protein and cowpea strain protein, the binding sites apparently being created by the juxtaposition of protein subunits. The presence of calcium ions in the medium promotes the polymerisation of the proteins of all four strains, presumably by "swamping out" the electrostatic repulsion between the charged protein subunits. This was evident particularly at high pH values where the protein would normally have depolymerised. In the case of both U2 and cowpea strain proteins, protein polymers arising in the presence of calcium at pH 6 were stabilised by the divalent cation even if the hydrogen ion concentration of the medium was subsequently reduced one hundred-fold. This was possibly a result of the Ca^{2+} ions linking adjacent subunits.

The binding of cations to BMV and to TCV, with the resultant stabilisation of these viruses, confirms that the binding of metal ions is a phenomenon not confined to TMV. In the light of the numerous plant, animal and bacterial viruses known to be stabilised by divalent cations, an investigation of the characteristics of cation binding by virus groups other than the tobamoviruses, bromoviruses, tymoviruses and tobraviruses (Durham et al., 1977a) would be a logical development of the present study. Cation binding appears to represent a third bonding factor in viruses, in addition to the mainly entropic protein-protein (Lauffer, 1975) and mainly ionic protein-nucleic acid bonds (Kaper, 1973). As such, cation binding obviously merits closer attention in virus purification, stability and reassembly studies.

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APPENDIX 1

The correction for light scattering of an $OD_{260\ nm}$ reading obtained with a virus solution

The example given below illustrates the calculation of the corrected $OD_{260\ nm}$ of the TMV vulgare preparation used in Table 6. Purified virus was suitably diluted in I=0.05 phosphate buffer pH 7.0, which buffer medium was used as a blank for zeroing the spectrophotometer at 260 nm, and for obtaining blank OD readings at every 20 nm interval from 340 nm to 500 nm. OD readings at 260 nm, and at every 20 nm interval from 340 nm to 500 nm (i.e. the light scattering), were then obtained for the virus solution, and were adjusted for the blank readings (see Table A1 below).

TABLE A1

Data used for the light scattering correction

λ (nm)	log λ	OD of blank	OD of TMV solution	TMV OD readings adjusted for blank	log of adjusted OD readings
500	2.6990	0.011	0.020	0.009	$\bar{3}.9542$
480	2.6812	0.012	0.022	0.010	$\bar{2}.0000$
460	2.6628	0.011	0.023	0.012	$\bar{2}.0792$
440	2.6435	0.014	0.028	0.014	$\bar{2}.1461$
420	2.6232	0.010	0.027	0.017	$\bar{2}.2304$
400	2.6021	0.009	0.029	0.020	$\bar{2}.3010$
380	2.5798	0.008	0.032	0.024	$\bar{2}.3802$
360	2.5563	0.007	0.037	0.030	$\bar{2}.4771$
340	2.5315	0.008	0.046	0.038	$\bar{2}.5798$
260	2.4149	0.000	0.633	0.633	$\bar{1}.0031$[a]

[a]This value was obtained by extrapolation of the plot of log (adjusted OD) vs log λ.

To calculate the extent of light scattering at 260 nm, the log of adjusted OD readings was plotted against log λ, and the computed regression line (which had a correlation coefficient of 0.99 and a slope of -3.75) was extrapolated to a log λ value of 2.4149 (i.e. log λ for 260 nm). This yielded a corresponding log OD value of $\bar{1}.0031$, corresponding to an OD (i.e. a scattering) at 260 nm of 0.101. This value subtracted from the uncorrected $OD_{260\ nm}$ value of 0.633 yielded a corrected $OD_{260\ nm}$ value of 0.532.

APPENDIX 2

The determination of virus-bound calcium by the sedimentation method

The example given below illustrates the determination of calcium bound to TMV vulgare at pH 7.0 and pCa 2.7 (see Fig. 5). TMV was dialysed against pH 7.0 imidazole buffer at I=0.16, and the concentration was then determined spectrophotometrically to be 10.92 mg TMV/ml. This virus solution was then treated as in section D of Chapter 3, the experimental protocol as well as the results obtained being summarised in Table A2 below.

TABLE A2

The determination of calcium bound to TMV at pH 7.0 and pCa 2.7

Before centrifuging						
Tube no.	Vol. TMV soln. (μl)	Vol. buffer added (μl)	Vol. 10^{-2}M calcium added (μl)	Final [TMV] (mg/ml)	Final [TMV] (μM TMVP)	Final $[Ca^{2+}]$ (μM)
1	0	800	200	0	0	2000
2	100	700	200	1.09	59.2	2000
3	200	600	200	2.18	118.3	2000
4	300	500	200	3.28	177.6	2000
5	400	400	200	4.37	236.7	2000
6	500	300	200	5.46	295.9	2000

Table A2 is continued overleaf.

Table A2 (continued)

	After centrifuging		
Tube No.	^{45}Ca in 250 μl supt. (cpm)[a]	$[Ca^{2+}]$ (μM)	$\Delta [Ca^{2+}]$ (μM)
1	52291	2000	-
2	51600	1970	30
3	50091	1920	80
4	48686	1860	140
5	47493	1820	180
6	45780	1750	250

[a]Each value represents the average of 5 separate counts.

The computed regression line of the plot of Δ[Ca]vs. virus concentration had a correlation coefficient of 0.99 and a slope, equivalent to Ca^{2+} ions bound per TMVP subunit, of 0.91.

APPENDIX 3

The plotting and standardising of hydrogen-ion titration curves

The example below shows how the titration curves of TMV vulgare in the absence of calcium, and in the presence of 1.5 mM and 27 mM Ca^{2+}, were calculated for Fig. 6. A 5 ml aliquot of TMV at a concentration of 5.48 mg/ml, equivalent to a total of 1.49 μmoles of TMVP, was used for this titration.

First, using 10 mM NaOH as titrant, a 5 ml volume of dialysate (50 mM KCl) was titrated as a blank from pH 4.5 to pH 8.5, the pH being recorded after each addition of 5 μl of titrant. This procedure was repeated using 5 ml dialysate plus a 100 μl 100 mM Ca^{2+}, and using 5 ml dialysate plus 200 μl 1 M Ca^{2+}. The addition of Ca^{2+} ions did not significantly alter the blank titration curve. The above procedure was then repeated using the aliquot of virus, the pH being recorded after each addition of 20 μl of titrant. After the initial titration of virus in the absence of calcium, the pH was restored to pH 4.5, 100 μl of 100 mM Ca^{2+} was added, the volume of titrant required to restore the pH to 4.5 noted as being equivalent to 0.12 H^+ per subunit, and the virus titrated to pH 8.5. The pH was then restored to pH 4.5, 200 μl of 1 M Ca^{2+} added, the amount of alkali needed to restore the pH to 4.5 noted as being equal to 0.45 H^+ per subunit, and the virus titrated for the third time. These titrations yielded plots of volume of 10 mM titrant added vs. pH for both blank and virus (Fig. A1).

From these plots, the total volume of 10 mM titrant was obtained for every 0.2 pH increment, and tabulated as in Table A3. At each pH, the volume of titrant (corrected for the blank volume) was converted to protons titrating per subunit, 149 μl of 10 mM NaOH being equivalent to 1 H^+ per TMVP subunit for a solution containing 1.49 μmoles of TMVP. These

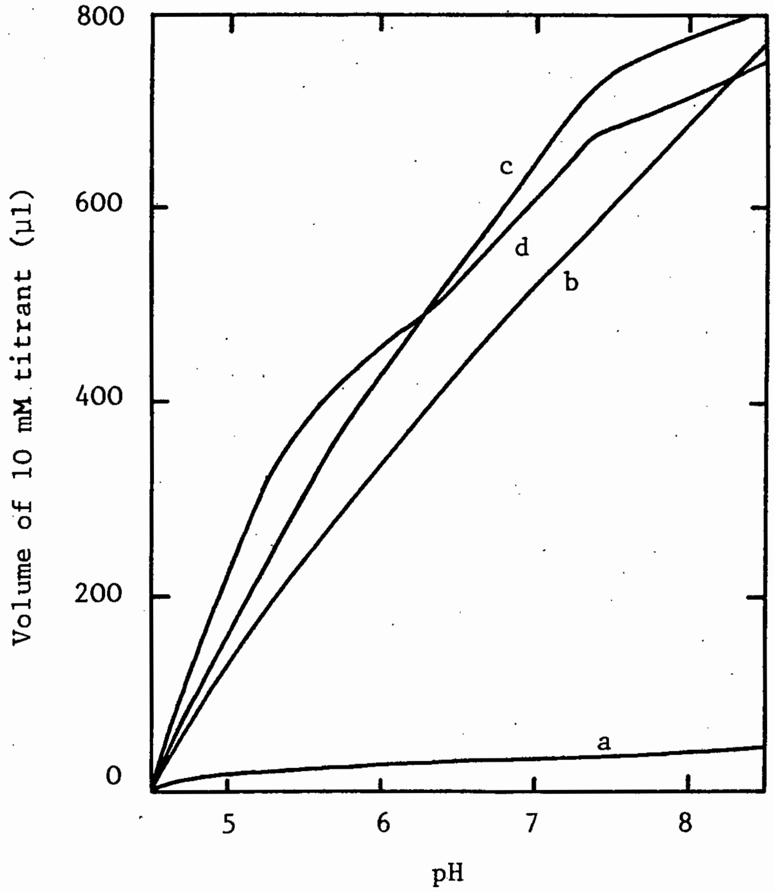

Figure A1. Titration plots (reduced from the original) of volume of 10 mM titrant added vs. pH for the 50 mM KCl blank (a), for TMV without added calcium (b), and for TMV in the presence of 1.5 mM Ca^{2+} (c) and 27 mM Ca^{2+} (d).

figures of H^+ per subunit were then adjusted for the proton displacement caused by the addition of calcium at pH 4.5, and plotted to obtain Fig. 6A. The curves in Fig. 6 B were obtained by simple subtraction at each pH of the adjusted H^+ per subunit figures for TMV without added calcium from those of TMV with calcium. These curves were then accurately aligned vertically using proton displacement figures obtained by the addition of calcium to aliquots of TMV adjusted to various pH values, and converting to H^+ per subunit the volumes of alkali required to restore the original pH value.

TABLE A3

The standardisation of hydrogen-ion titration curves of TMV

pH	Blank titrant vol.(µl)	TMV without added Ca^{2+}			TMV + 100 µl 100 mM Ca^{2+}				TMV + 210 µl 1M Ca^{2+}			
		Titrant vol.(µl)[a]	Corrected vol.(µl)[b]	H^+ per subunit[c]	Titrant vol.(µl)[a]	Corrected vol.(µl)[b]	H^+/subunit Calc.[c]	H^+/subunit Adj.[d]	Titrant vol.(µl)[a]	Corrected vol.(µl)[b]	H^+/subunit Calc.[c]	H^+/subunit Adj.[d]
4.5	0	0	0	0	0	0	0	0.12	0	0	0	0.57
4.6	1	39	38	0.26	45	44	0.30	0.42	61	60	0.40	0.97
4.8	4	92	88	0.59	109	105	0.70	0.82	153	149	1.00	1.57
5.0	8	141	133	0.89	174	166	1.11	1.23	237	229	1.54	2.11
5.2	11	186	175	1.17	240	229	1.54	1.66	316	305	2.05	2.62
5.4	14	226	212	1.42	291	277	1.86	1.98	370	356	2.39	2.96
5.6	18	266	248	1.66	344	326	2.19	2.31	412	394	2.64	3.21
5.8	21	306	285	1.91	392	371	2.49	2.61	442	421	2.83	3.40
6.0	24	346	322	2.16	442	418	2.81	2.93	464	440	2.95	3.52
6.2	26	386	360	2.42	486	460	3.09	3.21	490	464	3.11	3.68
6.4	29	425	396	2.66	530	501	3.36	3.48	518	489	3.28	3.85
6.6	31	461	430	2.89	574	543	3.64	3.76	551	520	3.49	4.06
6.8	33	498	465	3.12	616	583	3.91	4.03	586	553	3.71	4.28
7.0	35	530	495	3.32	663	628	4.21	4.33	618	583	3.91	4.48
7.2	36	564	528	3.54	704	668	4.48	4.60	656	620	4.16	4.73
7.4	38	596	558	3.74	738	700	4.70	4.82	689	651	4.37	4.97
7.6	39	630	591	3.97	759	720	4.83	4.95	702	663	4.45	5.05
7.8	40	664	624	4.19	773	733	4.92	5.04	713	673	4.52	5.09
8.0	41	701	660	4.43	785	744	4.99	5.11	725	684	4.59	5.16
8.2	42	735	693	4.65	796	754	5.06	5.18	737	695	4.66	5.23
8.4	43	764	721	4.83	806	763	5.12	5.24	751	708	4.75	5.32
8.6	45	793	748	5.02	817	772	5.18	5.30	770	725	4.87	5.44

[a] Read directly off Fig. A1.

[b] Obtained by subtraction of the blank figure from the figure in the previous column.

[c] In this experiment 149 µl of 10 mM titrant ≡ 1 H^+ subunit.

[d] Adjusted by adding to each figure the quantity of H^+/subunit displaced by the addition of Ca^{2+} at pH 4.5.